

昭和 32 年 7 月 9 日 第三種 郵便物 認可
第 46 卷 第 6 号 昭和 34 年 6 月 25 日 発行
(毎月 1 回 25 日 発行)
The Journal of Biochemistry, published monthly by
the Japanese Biochemical Society, Tokyo, Japan.
Vol. 46, No. 6, June 25, 1959

Vol. 46, No. 6

June, 1959

THE JOURNAL OF BIOCHEMISTRY

EDITED FOR THE JAPANESE BIOCHEMICAL SOCIETY

EDITORIAL BOARD

SHIRO AKABORI	SHIGERU AKAMATSU
SHICHIRO AKIYA	FUJIO EGAMI
SABURO FUNAHASHI	KOOZOO KAZIRO
KATASHI MAKINO	TOMOO MIWA
ZIRO NIKUNI	NORIO SHIMAZONO
YUSUKE SUMIKI	HIROSHI TAMIYA
TYUNOSIN UKITA	ITARU WATANABE
HARUHISA YOSHIKAWA	

PUBLISHED MONTHLY

BY

THE JAPANESE BIOCHEMICAL SOCIETY

Tokyo University, Tokyo

AGENCIES

Charles E. Tuttle
Rutland, Vt.
U.S.A.

Maruzen Co., Ltd.
Tokyo
Japan

Santo Vanasio
Köln
Germany

PRINTED IN JAPAN

THE JOURNAL OF BIOCHEMISTRY was founded in 1922 by Prof. emeritus Dr. S. Kakiuchi and had been edited by him to volume 36 of the year of 1944. After the discontinuation during the War, this JOURNAL has been restarted by The Japanese Biochemical Society, following the serial number from volume 37 of the year of 1950.

This JOURNAL is collecting the original investigations on the subject in this country. It is published monthly, one volume per year.

Note to Contributors

All manuscripts should be carefully typewritten, triple-spaced, in English, French or German, and addressed to *The Editor, The Journal of Biochemistry, The Japanese Biochemical Society, c/o Department of Biochemistry, Faculty of Medicine, Tokyo University, Bunkyo-ku, Tokyo, Japan.*

Authors receive 50 reprints of their papers as a rule at cost; additional copies can be prepared by the order on cost accounts. All orders for reprints should be markedly noted on the article paper or in a sheet of paper attached to it. On submitting the paper, it is preferable to attach the non-traced charts, besides original drawings, for circulation to Editors.

On request from authors to contribute to JOURNAL, the *Directions for preparation of manuscripts*, including styles of abbreviations of symbols, terms, units, and periodicals will be sent immediately.

Subscription

Subscription Price. ¥3,600 (U.S. \$10.00) per volume, postage inclusive. Back issues are available for sale, from volume 1 up-to-date at ¥3,600 (U.S. \$10.00) per volume, postage inclusive.

Remittances should be made preferably by check or draft payable to *The Japanese Biochemical Society, c/o Department of Biochemistry, Faculty of Medicine, Tokyo University, Bunkyo-ku, Tokyo, Japan.*

Foreign subscription is treated by the agencies of *Charles E. Tuttle, Co., Rutland, Vermont, U.S.A., Santo Vanasio, Köln, Germany, and Maruzen Co., Ltd., Chuo-ku, Tokyo, Japan.*

CONTRIBUTIONS OF THE WOOD-WERKMAN REACTION TO THE TRICARBOXYLIC ACID CYCLE*

By TAMITARŌ MIZUNOYA

(From the Laboratory of Biochemical Physics, the Faculty of Agriculture,
Kyushu University, Fukuoka)

(Received for publication, March 27, 1958)

The tricarboxylic acid (TCA) cycle is not a mere terminal stage of the oxidation of carbohydrate, but a joint of the metabolic pathways of carbohydrate, fat and protein. The function may thus be considered to be a kind of "metabolic buffer" which regulates the synthesis and degradation of those substances. It may also be said to be another function of the cycle to capture the available energy, because an energy-rich compound, ATP, which provides energy to biological activities, is generated through the operation of the cycle. Considering these functions of the TCA cycle, it is expected that the metabolic flow of TCA cycle might be controlled by some mechanisms in response to alterations of the external conditions.

According to the suggestion given by Ochoa (1), malic enzyme is probably concerned with the maintenance and regulation of the level of dicarboxylic acids in the TCA cycle, thus controlling tissue respiration and metabolism. However it is also stated that malic enzyme perhaps does not make much contribution to the TCA cycle, because the activity of malic enzyme *in vivo* is no more than 1~2 per cent of that of malic dehydrogenase involved in the TCA cycle (2). The two ideas lead to opposite assessments of the contribution to the TCA cycle of the Wood-Werkman reaction, the mechanism of which may involve malic enzyme (3). It was also suggested (4) that ATP may participate in the mechanism of the Wood-Werkman reaction, but the detailed mechanism of the reaction does not affect our results because our theory takes into account of only the apparent phenomena of the reaction.

In the present paper, the relation between TCA cycle and Wood-Werkman reaction is examined from the viewpoint of the thermodynamics of irreversible processes, and contributions of the latter reaction to the cycle are discussed.

THEORETICAL

1. *Preliminary Considerations*—In a previous paper (5), a metabolic pathway with cyclic process like TCA cycle was expressed as a catalytic reaction

* A part of this work was read before the 7th Symposium on Enzyme Chemistry held in Nagoya, July 17, 1954.

with n -step cyclic process, *i.e.*,

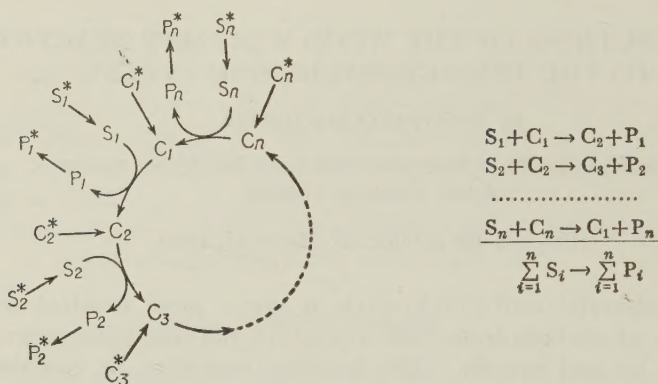
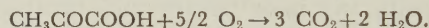


FIG. 1. A catalytic reaction with n -step cyclic process, which catalyzes the overall reaction from $\sum S_i$ to $\sum P_i$.

where C_i , S_i and P_i are the member of the catalytic cycle, the substrate and the product, respectively, of the i -th step of the cycle (Fig. 1). In the case of TCA cycle, the overall reaction from $\sum S_i$ to $\sum P_i$ corresponds to the complete oxidation of pyruvic acid, namely,



Actually some members of the TCA cycle are interconverted with some amino acids. Then the catalytic system has a variable amount of catalyst so that it can be called the "system of open catalytic cycle" in contrast to that of closed catalytic cycle which was treated in a previous paper (5) (Fig. 2). It is natural to think the cyclophorase-mitochondrial system as a system of open catalytic cycle.

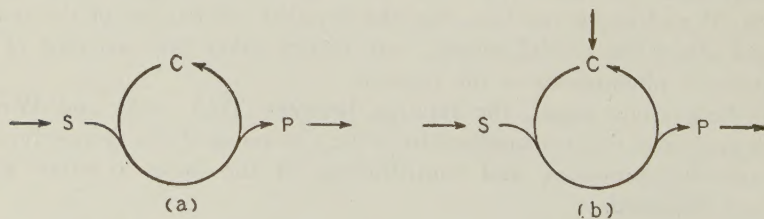
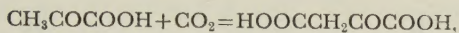


FIG. 2. (a) A closed catalytic cycle, (b) An open catalytic cycle.

The enzymes involved in the TCA cycle operate at the trace level of the substrates and in the course of the operation no intermediates accumulate in any measurable amount (6); in other words, this catalytic reaction proceeds in the stationary state. With some exceptions, metabolic flows are generally considered to be stationary. The Wood-Werkman reaction,



which can be taken as an interconversion between a catalyst C_i and a substrate S_i of the overall reaction from $\sum S_i$ to $\sum P_i$, also probably proceeds in the stationary state.

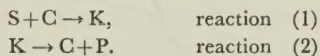
In the following we will discuss the stationary state of a system of open catalytic cycle with special reference to the TCA cycle.

2. *Mutual Relation of the Rates in the Stationary State*—In a previous paper (5), the mutual relation of the rates in the stationary state of a metabolizing system with catalytic cycle represented in Fig. 1 was derived as follows:

$$v_i = v_{i-1} + f_{ci} = f_{si} = -f_{pi} \text{ and } \sum_i f_{ci} = 0 \quad \begin{pmatrix} i=1, 2, \dots, n \\ i-1=n \text{ for } i=1 \end{pmatrix}, \quad (1)$$

where v_i is the net rate of the reaction from C_i to C_{i+1} in the i -th step of the catalytic cycle, defined by the change in concentration of the cycle component C_i per unit time. f_{ci} , f_{si} and f_{pi} are the net rates of the spatial or chemical transfers from the external region of the system into the internal one, of the catalyst (*i.e.* cycle components), of the substrate and of the product, respectively. These rates are defined by the changes in concentration of the corresponding species, C_i , S_i and P_i , within the system.

For simplicity, rewriting $\sum C_i$, $\sum S_i$ and $\sum P_i$ in the previous scheme (Fig. 1) as C, S and P, respectively, and introducing an intermediate complex K, a reaction of catalytic cycle (*i.e.* the complete oxidation of pyruvic acid by the TCA cycle) may be formulated in the simplest form (Fig. 3a):



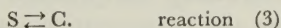
Applying the relation (1) to the simplified system represented in Fig. 3 a, we obtain

$$\begin{aligned} v_1 &= v_2 + f_c = f_s, \\ v_2 &= v_1 + f_k = -f_p, \end{aligned} \quad (2)$$

where v_1 and v_2 are the net rates of the reactions (1) and (2), respectively. Since K is an intermediate of the reaction, the catalytic system does not exchange K with its environment, namely, f_k is zero. Hence the relation (2) becomes

$$\begin{aligned} v_1 &= v_2 = f_s = -f_p, \\ f_c &= 0. \end{aligned} \quad (3)$$

On the other hand, the interconversion between the substrate and the catalyst (*i.e.* the Wood - Werkman reaction) is expressed as



Denoting by v_3 the net rate of the reaction (3) from S to C, the transfer rates of catalyst f_c and of substrate f_s may be rewritten as

$$\begin{aligned} f_c &= f'_c + v_3 = 0, \\ f_s &= f'_s - v_3, \end{aligned} \quad (4)$$

where f'_c and f'_s are the net rates of all the transfers, except for the reversible reaction (3), from the external region of the catalytic system into the internal one, of the catalyst (cycle components) and of the substrate,

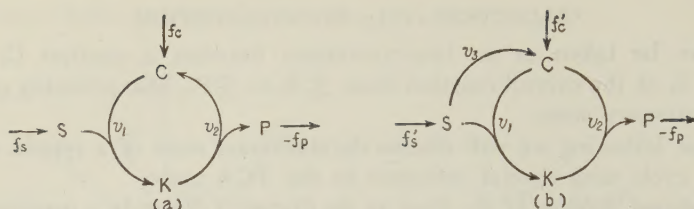


FIG. 3. A reaction system of open catalytic cycle (a) in the simplest form and (b) accompanied by the interconversion between the catalyst and its substrate.

respectively (Fig. 3b). Thus we obtain the following relations:

$$v_1 = v_2 = f'_s - v_3 = -f_p \equiv J_{ca}, \quad (5)$$

$$v_3 = -f'_c \equiv J_{an}, \quad (6)$$

and

$$f'_s = J_{ca} + J_{an}. \quad (7)$$

J_{ca} corresponds to the metabolic flow of the dissimulation (*i.e.* the *catabolic* flow) of pyruvic acid to carbon dioxide and water *via* the TCA cycle, while J_{an} to the metabolic flow of the assimilation (*i.e.* the *anabolic* flow) of pyruvic acid to some amino acids in the metabolic pool *via* the Wood-Werkman reaction (Fig. 4).

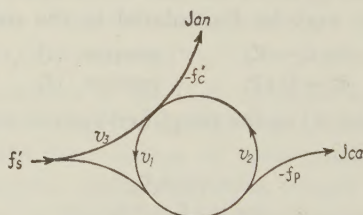


FIG. 4. J_{ca} is the catabolic flow of pyruvic acid to carbon dioxide and water *via* TCA cycle. J_{an} is the anabolic flow of pyruvic acid to amino acids *via* Wood-Werkman reaction. f'_s is the rate of supply of pyruvic acid and related to the two flows by $f'_s = J_{ca} + J_{an}$.

3. *Transition between Stationary States (I)*—From the relations (5) and (6), it is evident that the transfer rate $-f'_c$ of the catalyst and the reaction rate v_3 from S to C are mutually compensated in the stationary state of the system and that when the compensation does not occur ($v_3 \neq -f'_c$), the catalytic system is in a non-stationary state. We can also find two stationary states of the catalytic system, which have the stationary flow J_{ca} accompanied by the flow either $J_{an} = 0$ or $J_{an} \neq 0$. When the catalytic system which has been in the first stationary state ($J_{ca} \neq 0$ and $J_{an} = 0$) is compelled to accept a new condition of $f'_c \neq 0$, we may see two responses: if the reaction (3) can not take place the system will come into a non-stationary state, but if the reaction (3) is able to occur the system will shift from the first stationary

state to the second one ($J_{ca} \neq 0$ and $J_{an} \neq 0$). The catalytic system coexisting with enzymes involved in the reaction (3) can thus be always in the stationary state, whether the f'_c -value is zero or finite.

According to the Baldwin's book (7), in actual cases oxaloacetate drifts out of the system by spontaneous β -decarboxylation even in muscle which contains no oxaloacetic decarboxylase. In other words, $-v_3$ is fairly small but not zero. In minced muscle, where $f'_c = 0$, the rate of respiration, J_{ca} , slowly declines because $-v_3 \neq f'_c = 0$. "Presumably" says Baldwin, "the maintenance of a high rate of respiration in intact muscle requires the provision of a constant supply of some one or other of the catalytic dicarboxylic acids", namely, $-v_3 = f'_c \neq 0$ in our expression.

4. *Mutual Interference of the Two Stationary Flows*—In the relation (7) is shown the fact that the catabolic and anabolic flows interact complementarily with each other, that is to say, the increase in J_{an} is accompanied by the decrease in J_{ca} when pyruvic acid is constantly supplied to the system ($f'_s = \text{constant}$). Such a situation may well be represented by cancerous tissues, in which the synthetic activity of protein and nucleic acid may be naturally expected to increase abnormally. In fact, cancerous liver tissue incorporates C^{14} -alanine, *i.e.*, a precursor of pyruvic acid at a rate which is about 7 times higher than that of normal one (8). In addition, the pathway for synthesis of nucleic acid is suggested as follows: oxaloacetic acid $\rightarrow$ aspartic acid $\rightarrow$ orotic acid $\rightarrow$ uracil $\rightarrow$ nucleic acid (9). It would be reasonable then to expect a decrease in the amount of catabolic flow of pyruvic acid in cancerous tissue. This is confirmed by the finding that the enzyme system involved in the first step of the TCA cycle is injured in cancerous tissue (10).

In order to elucidate contributions of the Wood-Werkman reaction to the TCA cycle, it is of interest to consider in more detail the mutual interference of both catabolic and anabolic flows of pyruvic acid.

It was shown in a previous paper (5) that a metabolizing system with cyclic processes dissipates the available energy at the following rate:

$$T\sigma = \sum_i v_i A_i + \sum_m f_m A_m, \quad (8)$$

where A_i and A_m are the chemical affinities of the reaction of the i -th step of the cycle and of the chemical transfer of the species m , respectively. If the transfer of species m is a process of diffusion type, A_m is the driving force for the spatial transfer.

Using the relations (5)–(7), the energy dissipation $T\sigma^0$ in the stationary state with two metabolic flows J_{ca} and J_{an} may thus be expressed as*

$$T\sigma^0 = J_{ca} X_{ca} + J_{an} X_{an} \quad (9)$$

where

$$\begin{aligned} X_{ca} &\equiv A_1 + A_2 + A_s - A_p, \\ X_{an} &\equiv A_3 + A_s - A_e. \end{aligned} \quad (10)$$

* In Eqn. (9) we must take care of the nature of quantities, whether chemical or spatial. However, in Eqn. (10) both quantities can be taken as members of J_{ca} , J_{an} , X_{ca} and X_{an} .

X_{ca} and X_{an} are the driving forces for the catabolic and anabolic flows, respectively.

For simplicity it is assumed that there is no interference among the individual reactions and/or transfers. When the system is nearly in equilibrium, the rates of the above reactions and transfers are linearly related to the chemical affinities introduced by de Donder as follows:

$$\begin{aligned} v_1 &= L_1 A_1 = L_1(\mu_s + \mu_c - \mu_k), \\ v_2 &= L_2 A_2 = L_2(\mu_k - \mu_c - \mu_p), \\ v_3 &= L_3 A_3 = L_3(\mu_s - \mu_c), \\ f'_c &= L_c A_c = L_c(\mu_c^* - \mu_c), \\ f'_s &= L_s A_s = L_s(\mu_s^* - \mu_s), \\ f'_p &= L_p A_p = L_p(\mu_p^* - \mu_p), \end{aligned} \quad (11)$$

where μ_c , etc., and μ_c^* , etc., are the chemical potentials in the system and in its environment, respectively, provided that the transfer is spatial. If the transfer is chemical the latter is the chemical potential of the precursor of the corresponding species. L_1 , etc., are phenomenological coefficients, which are not negative. Hence the expression (10) reduces to

$$\begin{aligned} X_{ca} &= \mu_s^* - \mu_p^*, \\ X_{an} &= \mu_s^* - \mu_c^*. \end{aligned} \quad (12)$$

Using the relations (5)–(7) and (11), these forces are expressed by

$$\begin{aligned} X_{ca} &= R_{ca} J_{ca} + R_s J_{an}, \\ X_{an} &= R_s J_{ca} + R_{an} J_{an}, \end{aligned} \quad (13)$$

where

$$\begin{aligned} R_{ca} &= R_1 + R_2 + R_s + R_p, \\ R_{an} &= R_3 + R_s + R_c, \\ R_i &= 1/L_i > 0 \quad (i = 1, 2, 3), \\ R_m &= 1/L_m > 0 \quad (m = c, s, p). \end{aligned} \quad (14)$$

R_i and R_m are the resistances to the reaction i and to the transfer of species m , respectively. The former is related to the degree of activation of the reaction, while the latter to that of permeability of the boundary of the system for the species. R_{ca} and R_{an} are the resistances to the stationary flows J_{ca} and J_{an} , respectively. All the resistances are positive definite.

From Eqns. (13) the following constant ratios are found:

$$\begin{aligned} \rho_{ca} &\equiv \left(\frac{X_{ca}}{X_{an}} \right)_{J_{an}=0} = \frac{R_{ca}}{R_s} > 1 && \text{in the case of } J_{an}=0, \\ \rho_{an} &\equiv \left(\frac{X_{an}}{X_{ca}} \right)_{J_{ca}=0} = \frac{R_{an}}{R_s} > 1 && \text{in the case of } J_{ca}=0. \end{aligned} \quad (15)$$

ρ_{ca} and ρ_{an} are the resistances to the stationary catabolic and anabolic flows, respectively, per unit resistance to the supply of the substrate, i.e., of pyruvic acid. Inserting these constant ratios into Eqns. (13), we obtain

$$\begin{aligned} J_{ca} &= (\rho_{an} X_{ca} - X_{an}) / R_s (\rho_{ca} \rho_{an} - 1), \\ J_{an} &= (\rho_{ca} X_{an} - X_{ca}) / R_s (\rho_{ca} \rho_{an} - 1). \end{aligned} \quad (16)$$

These relations can be expressed more briefly in the form of

$$\begin{aligned} J_{ca} &= \lambda_{ca} X_{ca} + \lambda X_{an}, \\ J_{an} &= \lambda X_{ca} + \lambda_{an} X_{an}, \end{aligned} \quad (17)$$

with the notations

$$\begin{aligned} \lambda &\equiv -1/R_s(\rho_{ca}\rho_{an}-1) < 0, \\ \lambda_{ca} &= -\rho_{an}\lambda > 0, \\ \lambda_{an} &= -\rho_{ca}\lambda > 0. \end{aligned} \quad (18)$$

The coefficient λ is related to the mutual interference of the two stationary flows. The quantity $\rho_{ca}\rho_{an}$ depends on the ratios of R_i , R_c and R_p to R_s . The relation between λ and $\rho_{ca}\rho_{an}$ is expressed by a kind of hyperbola where the asymptotes are $\rho_{ca}\rho_{an}=1$ and $\lambda=0$. If R_s vanishes, then $\rho_{ca}\rho_{an}$ is infinite, the interference coefficient λ is zero but both λ_{ca} and λ_{an} are finite. In this case, the two stationary flows are independent with each other. While, if $\rho_{ca}\rho_{an}$ is finite both flows appear interfering with each other. The mutual interference depending on the permeability thus appears between the two stationary flows even though the rates of individual reactions and transfers are independent of one another. Since the λ is negative, the interference gives a negative effect to the stationary flows. When $\rho_{ca}\rho_{an}$ approaches to unity, the absolute value of λ is large but finite*. Furthermore, when R_s is very much larger than the other resistances, both flows interfere much more intensively with each other and are in the relation of

$$J_{ca} = -J_{an} = \lambda(\mu_c^* - \mu_p^*). \quad (19)$$

In other words, when the resistance to the supply of pyruvic acid into the system is much larger than that to the supplies of other species and also that to the reaction within the system, the flow from some amino acids in the metabolic pool to carbon dioxide and water may take place through the Wood-Werkman reaction and the TCA cycle (Fig. 5). Thus amino acids instead of pyruvic acid are completely oxidized through the TCA cycle. On the other hand, it is evident from Eqns. (17) and (18) that the two flows and their interference are inversely proportional to the resistance to supply of the substrate. It is, therefore, concluded that the resistance to supply of pyruvic acid must be as small as possible under the conditions stated above in order that the flow of complete oxidation of

* From Eqns. (14) and (15),

$$\rho_{ca} = 1 + \frac{R_1 + R_2 + R_p}{R_s} \quad \text{and} \quad \rho_{an} = 1 + \frac{R_3 + R_c}{R_s}.$$

Hence,

$$\rho_{ca}\rho_{an} = 1 + \frac{R_1 + R_2 + R_3 + R_p + R_c}{R_s} + \frac{(R_1 + R_2 + R_p)(R_3 + R_c)}{R_s^2}.$$

If we can neglect the term of second order in respect to $1/R_s$, the following relation is found:

$$R_s(\rho_{ca}\rho_{an}-1) = R_1 + R_2 + R_3 + R_p + R_c.$$

When the right-hand of this equation is finite, the interference coefficient λ is also finite. In this case, $\rho_{ca}\rho_{an}-1$ must be infinitesimal of the same order as the inverse of R_s . As to this footnote, the author's thanks are especially due to Prof. M. Sugita for his kind advice.

amino acids may be large.

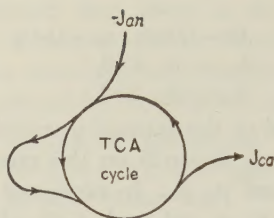


FIG. 5. The metabolic flow of complete oxidation of amino acids which is expressed by the relation $J_{ca} = -J_{an}$.

5. *Transition between Stationary States (II)*—It was previously stated that the flow of the TCA cycle can be in two kinds of stationary states accompanied by the flow of $J_{an} = 0$ or $J_{an} \neq 0$ and the transition is able to occur between these states. From Eqn. (17) the metabolic flow of the TCA cycle is rewritten as

$$J_{ca} = -\left(\rho_{an} - \frac{1}{\rho_{ca}}\right) \Lambda X_{ca} - \frac{J_{an}}{\rho_{ca}} \quad \text{for } J_{an} \neq 0. \quad (20)$$

In the case of $J_{an} = 0$ where the Wood-Werkman reaction is not taking place, we obtain

$$J_{ca} = -\left(\rho_{an} - \frac{1}{\rho_{ca}}\right) \Lambda X_{ca} \quad \text{for } J_{an} = 0. \quad (21)$$

Using Eqns. (20) and (21), the increase in the amount of flow of the TCA cycle J_{ca} , caused by the transition from a stationary state with $J_{an} = 0$ to another one with $J_{an} \neq 0$, is given by

$$\begin{aligned} \Delta J_{ca} &= J_{ca} \text{ (for } J_{an} \neq 0) - J_{ca} \text{ (for } J_{an} = 0) \\ &= -J_{an}/\rho_{ca}. \end{aligned} \quad (22)$$

The metabolic flow of the TCA cycle J_{ca} is estimated as the number of moles of pyruvic acid completely oxidized per unit time. In one operation of the TCA cycle, one molecule of pyruvic acid is completely oxidized and fifteen molecules of ATP are generated. Hence the energy captured (or ATP generated) ϵ in the TCA cycle per unit time is proportional to the flow of TCA cycle J_{ca} . Using Eqn. (22) the increase of the energy captured $\Delta\epsilon$ per unit time, caused by the transition from the stationary state with $J_{an} = 0$ to the one with $J_{an} \neq 0$, is given by

$$\Delta\epsilon = \alpha \cdot \Delta J_{ca} = -\frac{\alpha}{\rho_{ca}} \cdot J_{an}, \quad (23)$$

where α is a proportional coefficient. Taking the number of moles of ATP as the unit of energy captured, the coefficient α becomes fifteen. Eqn. (23) shows that the metabolic flow of TCA cycle and hence the energy captured (or ATP generated) in the cycle decrease in proportion to the metabolic flow of Wood-Werkman reaction J_{an} , while they increase if the latter flow goes in the opposite direction.

CONCLUSIONS AND DISCUSSIONS

1. *Contributions of Wood-Werkman Reaction to TCA Cycle*—It was previously shown (5) that only when the total concentration of the catalyst is kept constant, the catalytic reaction can proceed in the stationary state. In actual cases, in spite of the fact that some members of the TCA cycle are interconverted with some amino acids, *i.e.*, that the amount of the catalyst (the members of TCA cycle) is able to vary, the catalytic reaction (the complete oxidation of pyruvic acid) proceeds in the stationary state. This discordance would be related to contributions of the Wood-Werkman reaction to the TCA cycle in the following way.

As shown in this paper, the interconversions among the members of TCA cycle and some amino acids in the metabolic pool are compensated by the Wood-Werkman reaction. The metabolic flow of TCA cycle may thus be maintained in the stationary state. If the interconversions take place the stationary flow of TCA cycle shifts to the other stationary state accompanied by the stationary flow of the Wood-Werkman reaction. In the latter stationary state, the two stationary flows interfere complementarily with each other, and when the supply of pyruvic acid into the system is prevented or diminished, amino acids are completely oxidized through the TCA cycle accompanied by the Wood-Werkman reaction. The TCA cycle is, therefore, able to operate effectively as a sort of "metabolic buffer" in cooperation with the Wood-Werkman reaction. This conclusion agrees with the Ochoa's suggestion stated above. Further, when the transition occurs between the two stationary states, the stationary flow of TCA cycle changes in proportion to the stationary flow of Wood-Werkman reaction. Hence the change in the amount of energy captured (or the quantity of ATP generated) in the TCA cycle is controlled by the latter flow and its direction. In other words, the TCA cycle may be regarded as an "energy capturing system" controlled by the Wood-Werkman reaction. As stated above, it can be concluded that the two functions of the TCA cycle, that is, the energy capture and metabolic buffer actions, may reveal themselves in connection with the Wood-Werkman reaction.

2. *Possibility of Generalization*—In the present paper, a theory has been developed with special reference to the TCA cycle and the Wood-Werkman reaction. This may be generally applicable to reaction systems of open catalytic cycle where the amount of catalyst is variable. For example, the members of the dicarboxylic acid cycle are also interconvertible with some amino acids, and one of those, *i.e.*, acetic acid is the substrate to be oxidized completely through the cycle. In this case, the interconversion between the catalyst and its substrate, *i.e.*, the reaction (3) is considered to be a process of diffusion type rather than that of reaction type. We may also be able to find an example in the behavior of enzyme *in vivo*. It may possibly be thought that the degradation or denaturation of enzyme molecules

in vivo is compensated by means of their adaptative formation induced by the substrate. Furthermore, it may be considered that the degradation of foods is "catalyzed" by the organism and the dissimilation of the living body is compensated by the assimilation of foods. It may thus be expected that the reaction system of open catalytic cycle might have some mechanisms which compensate variations of the amount of catalyst in the stationary state of the system.

SUMMARY

The TCA cycle *in vivo* can be regarded as a kind of reaction system of open catalytic cycle in view of the fact that the members of cycle are interconvertible with some amino acids. In a previous paper, the author have developed a general theory of a metabolizing system with cyclic process, and now it is applied to a reaction system of open catalytic cycle with special reference to the TCA cycle. It is shown that the interconversion between the members of TCA cycle and some amino acids is compensated by the reversible Wood-Werkman reaction, by means of which the metabolic flow of TCA cycle can be maintained in the stationary state.

We have thus two kinds of the stationary states of the TCA cycle either accompanied or not by the stationary anabolic flow of pyruvic acid to some amino acids through the Wood-Werkman reaction. In contrast with the latter flow, the flow of TCA cycle can be called the catabolic flow of pyruvic acid to carbon dioxide and water.

When pyruvic acid is constantly supplied into the system the two flows interfere complementarily with each other. The equations are derived which represent the mutual interference of the two stationary flows. The interference depends on the resistance to the supply of pyruvic acid into the system. When this resistance is much larger than all other ones, the most intensive interference appears and consequently amino acids instead of pyruvic acid are completely oxidized through the operation of the TCA cycle accompanied by the Wood-Werkman reaction.

When the metabolic flow of TCA cycle shifts from a stationary state without the flow of Wood-Werkman reaction to another one accompanied by the latter flow, the former flow and hence the energy captured in the cycle decrease in proportion to the latter flow, while they increase provided that the latter flow goes in the opposite direction.

The present theory thus leads to the following conclusions: 1) The TCA cycle may be able to operate effectively as a "metabolic buffer" in cooperation with the Wood-Werkman reaction. 2) The TCA cycle may be regarded as an "energy capturing system" controlled by the Wood-Werkman reaction.

The author expresses his sincere appreciation to Prof. K. Oomori and Ass. Prof. G. Tomita of this laboratory for their advice and encouragement, and to Prof. S. Akabori of Osaka University for his interest and encouragement in the work. Grateful

acknowledgement is made to Prof. M. Sugita of Hitotsubashi University for his repeated inspection of the manuscript and many valuable advices. The author has been greatly encouraged by his constant interest and helpful discussions.

REFERENCES

- (1) Ochoa, S., Veiga Salles, J. B., and Ortiz, P. J., *J. Biol. Chem.*, **187**, 863 (1950)
- (2) Okunuki, K., *Chemistry of Fermentation (in Japanese)*, Kyoritsu Publ. Co., Tokyo, p. 188 (1951)
- (3) Veiga Salles, J. B., and Ochoa, S., *J. Biol. Chem.*, **187**, 849 (1950)
- (4) Utter, M. F., *J. Biol. Chem.*, **188**, 847 (1951)
- (5) Mizunoya, T., *J. Biochem.*, **46**, 535 (1959)
- (6) Green, D. E., *Enzymes and Enzyme Systems*, ed. by Edsall, J. T., Harvard Univ. Press, Cambridge, p. 28 (1951)
- (7) Baldwin, E., *Dynamic Aspects of Biochemistry*, Cambridge Univ. Press, Cambridge, 2nd ed., p. 450 (1952)
- (8) Zamecnik, P. C., Fantz, I. D., Jr., Loftfield, R. B., and Stephenson, M. L., *J. Biol. Chem.*, **75**, 299 (1948)
- (9) Mitchell, H. K., and Houlahan, M. B., *Fed. Proc.*, **6**, 506 (1947); Plentl, A. A., and Schoenheimer, R., *J. Biol. Chem.*, **153**, 203 (1944); Bergstrom, S., Arvidson, H., Hammarsten, E., Eliasson, N. A., Reichard, P., and Ubisch, H. V., *J. Biol. Chem.*, **177**, 495 (1949)
- (10) Potter, V. R., and Le Page, G. A., *J. Biol. Chem.*, **177**, 237 (1949); Potter, V. R., Le Page, G. A., and Klug, H. L., *J. Biol. Chem.*, **175**, 619 (1948); Potter, V. R., Pardee, A. B., and Lyle, G. G., *J. Biol. Chem.*, **176**, 1075 (1948)

DIFFERENTIAL MICRODETERMINATION OF BILE ACIDS IN BILE BY PAPER CHROMATOGRAPHY

BY NOBUYUKI WATANABE

(From the 1st Surgical Department, Faculty of Medicine,
Kyushu University, Fukuoka)

(Received for publication, July 29, 1958)

For the purposes of studying bile acid metabolism and gall stone formation, several methods for the separation of bile acids, *i.e.*, counter current distribution, column absorption chromatography, column partition chromatography, and paper partition chromatography (p.p.c.) (1-7) have been published. Sjövall (8-10) and Inutsuka (11) have independently refined a paper chromatographic method for quantitative work.

The quantitative determination of bile acids by use of ultraviolet absorption in sulfuric acid has been studied by Bandow (12), Kier (13) and Mosbach *et al.* (14). Recently Eriksson and Sjövall (15) and Kazuno (16) also have studied this method in much more detail.

The present paper deals with, first, the complete quantitative separation of free, glycine-conjugated and taurine-conjugated bile acids from lipids in bile which interfered with the determination, either by two-step p.p.c. or by extraction with 50 per cent ethanol and petroleum ether. Secondly, we discuss the separation of taurine-conjugates by p.p.c. effected by the use of a new solvent system. And thirdly, we consider the quantitative microdetermination of the conjugated and free bile acids separated by p.p.c. by means of ultraviolet absorption in 65 per cent sulfuric acid. Further investigations of the conditions for the determination of bile acids revealed the possibility of determining conjugated-chenodeoxycholic and deoxycholic acids in a solution containing both of them. The heating conditions of free and conjugated cholic acid were investigated and it was found that the heating condition at 28° for 60 minutes was better than that at 60° for 15 minutes.

With this method, free, glycine-conjugated and taurine-conjugated bile acids were determined simply and accurately, excluding the influence of some interfering substances as lipids in bile.

I. Separation of Free and Conjugated Bile Acids by Paper Chromatography

EXPERIMENTALS AND DISCUSSION

A. Reagents and Filter Paper:

1. *Reagents:* (a) *Bile Acids*—Chenodeoxycholic (CHD), deoxycholic (DE), cholic (C), and glycocholic (GC) acids were prepared according to the

standard methods and showed the melting points reported in the literature. Glycochenodeoxycholic (GCHD) acid was kindly supplied by Prof. T. Shimizu (Faculty of Medicine, Okayama University). Glycodeoxycholic acid (GDE) was prepared from rabbit bladder bile.

(b) *Solvents*—Ethanol (96 per cent), methanol, glacial acetic acid, isoamyl formate and *n*-heptane were all redistilled. Ethyl butyrate was shaken with concentrated sulfuric acid, washed with diluted NaOH and water, dried over K_2CO_3 and distilled.

(c) 50 per cent aqueous methanol (pH:12) prepared by mixing equal volumes of 1/80 *N* NaOH solution and methanol before use.

2. *Preparation of Paper*: "Toyo" filter paper No. 50, No. 51 or No. 53 washed with ethanol, was used. The filter paper No. 53 which was treated with diluted HCl contained less ashes and ferric-ion. The filter paper was cut (30×40 cm.) and divided lengthwise into three sections: Section A (6 cm.), Section B (12 cm.), and Section C (12 cm.). In Section B, five spots were marked at the intervals of 1.5 cm. Section C was used as a "blank".

3. *Washing of the Filter Paper*: To remove "dirt", the filter paper was washed with ethanol in an ordinary descending paper chromatographic vessel for 72 hours.

4. *Determination of the Position and Elution of the Separated Bile Acids*: For the determination of the position of the separated bile acids, pure bile acids (10–20 μ g.) and the same volume of bile solution as that which was spotted on one spot in Section B were spotted in Section A, subjected to p.p.c. The spots in Section A were revealed by spraying with a solution of phosphomolybdic acid (30 per cent in ethanol) followed by heating at 100–120°. The horizontal segments of Sections B and C corresponding to the spots of each bile acid on Section A were cut out. This segment contained the 5 spots of separated bile acid. One end of the segment was clamped between two microscopic slides and eluted with ethanol in the flat bottomed test tube with glass stopper (4×10 cm.) for 24 hours.

B. *Behavior of Bile Acids and Lipids in Paper Chromatography by Use of Various Concentrations of Methanol*—If the bile sample was subjected to p.p.c. with either TEW-solvent or EBH-solvent, two spots of Lipid-A and Lipid-B were found. The spots of free dihydroxycholic acids (DIH) and C acid were overlapped with the spots of Lipid-B which formed a sharp triangle. These lipids interfered with the detection of free bile acids on the chromatogram and showed considerable ultraviolet absorption in the same condition for quantitative determination of bile acid. To remove these interfering lipids, Inutsuka (11) performed two-step p.p.c. First, the p.p.c. with 40 per cent aqueous methanol (pH 7) was performed. The solvent was allowed to rise to a height of 6 cm., while the lipids remained at the starting spots. Then, after drying and cutting the paper at the height of 1 cm. from the starting line, the p.p.c. was proceeded with the TEW-solvent. Thus, Inutsuka separated free bile acids and glycine-conjugates, excluding the influence of lipids in bile.

But with this method, part of the free DIH acids remained at the starting spot, so it could not be applied to the quantitative determination of free DIH acids. Therefore, p.p.c. was performed by changing the concentration of aqueous methanol to 50, 55, 60, 70 and 80 per cent. When concentration of methanol increased to more than 60 per cent the DIH acids ascended smoothly, but at the same time lipids also ascended. Consequently with this method it was quite difficult to completely separate lipids from the DIH acids.

In Lipid-A, cholesterol was found. In Lipid-B, phosphorus was found. This fact suggested that phospholipid, probably lecithin, existed in Lipid-B.

C. The Effect of Varying the pH of 50 Per Cent Methanol on the Behavior of Bile Acids and Lipids—The changes in p.p.c. brought about by changing the pH of 50 per cent methanol solutions were as follows. (1) pH: 3—Both DIH acids and lipids ascended. (2)—As the pH was adjusted to 8, 9, 10, 11 and 12, DIH acids and all other bile acids ascended smoothly; while lipids remained at the starting spot. When the pH rose to more than 12, none of the DIH acids were found at the starting spot.

D. Paper Chromatography of Glycine-conjugated and Free Bile Acids—In the method reported by Beyreder *et al.* (2), the solvent system composed of toluol/glacial acetic acid/water (5:5:1) was equilibrated and the upper layer (TEW-solvent) was used for the experiment. Before p.p.c. the filter paper was equilibrated overnight with the vapour of 70 per cent acetic acid. The ascending p.p.c. was run to a height of 30 cm. for 5 hours at 15–25°.

E. Paper Chromatography of Taurine-, Glycine-conjugated and Free Bile Acids—Methods for the separation of taurine-conjugates by p.p.c. have been published only by Sjövall (5, 7).

In the author's method, ethyl butyrate (or isoamyl formate):heptane (90:10) was used as moving phase and 70 per cent acetic acid as stationary phase. Equal parts of the phases were equilibrated before use, at the temperature 25–30° and the upper layer (EBH-solvent) was used.

The p.p.c. run to 30 cm. (12–15 hours) with EBH-solvent at 25–30° showed good separation not only of tauro-DIH acids and tauro-C acid but also of free-DIH acids, GDIH acids, and GC acid, if lipids had been removed. However, the R_f values of the GDIH acids and free C acid were approximately the same, and a good separation could not be attained by this system in p.p.c. to 30 cm.

When this EBH-solvent was used, the spots appeared well defined with no tailing, so the equilibration of the filter paper with this solvent before running the p.p.c. could be omitted.

F. Preparation of Bile Sample Solution—Human gall bladder bile obtained by laparotomy was used.

1. Crude Bile Sample Solution: 1–4 ml. of bladder bile was used. To remove the protein completely, the bile was added to ethanol and heated. This solution was filtered quantitatively and evaporated on a water bath. The resulting sirup was transferred to 10 ml. volumetric flask and ethanol

was added up to the mark.

2. *Lipid-free Bile Sample*: If the lipid concentration in bile was comparatively high, as in normal human gallbladder bile and dog bile, the separation of the lipids from bile acids by two-step p. p. c. was unsatisfactory and the lipids had to be removed by a preliminary procedure. Utilizing the fact that the lipids in bile are quite insoluble in 50 per cent aqueous ethanol (or methanol), 5 ml. of crude bile sample solution mentioned above, was pipetted into a 25 ml. centrifuge tube and 3.5 ml. of petroleum ether was added, mixing well. After the addition of about 5 ml. of distilled water, this solution was shaken vigorously, centrifuged, separated into two layers, and the upper petroleum ether layer removed. This procedure was repeated several times till the white layer between two layers faded away. The aqueous solution was evaporated to dryness on a water bath, and cooled. 2 ml. of ethanol was pipetted into this residue and it was used as "lipid-free bile sample". When the concentration of ethanol was more than 60 per cent (pH 7), it was difficult to remove the lipids completely by the above mentioned procedure, and in such cases, the lipids were still existent all the time.

G. *Paper Partition Chromatography by This Method and R_f Values of Bile Acids.*

1. The suitable volume of crude bile sample solution necessary for the clean separation of bile acids (10–20 μ g.) was estimated by preliminary p. p. c. Five starting spots, each containing this suitable volume of crude bile sample solution, were spotted in Section-B with a micropipette.

TABLE I
 R_f Values of Bile Acids in Ascending P.P.C. with Various Solvent

	50% aqueous methanol (pH 12)	TEW-solvent	Two-step- p. p. c.	EBH-solvent
DIH acid	0.78	0.75	0.72	0.91
C acid	0.90	0.61	0.58	0.71
G-DIH acid	0.85	0.45	0.46	0.67
G-C acid	0.83	0.18	0.30	0.58
T-DIH acid	0.93	0	0.23	0.32
T-C acid	0.93	0	0.23	0.23
Lipid-A	0	0.96	0	
Lipid-B	0	0.91 0.85 0.71	0	

G: glyco-, T: tauro-, DIH: dihydroxycholic acids (chenodeoxycholic acid and deoxycholic acid) and C: cholic acid.

2. To remove the lipids in the bile, p.p.c. (50 per cent aqueous methanol, pH 12) was run to the height of 6 cm. The bile acids ascended with their definite R_f and the lipids remained at the starting spot. After drying, the paper was cut 1 cm. from the starting spot which contained the lipids. If the lipids were previously removed by extraction, this procedure was unnecessary.

3. To separate free bile acids and GDIH acids, the upper part of the filter paper was paper-chromatographed with TEW-solvent (30 cm. for 5 hours).

4. To separate glycine- and taurine-conjugates, the upper part of the filter paper was paper-chromatographed with the EBH-solvent at 25–30° (30 cm., for 12–15 hours). R_f values of each bile acid are shown in Table I.

II. Quantitative Microdetermination of Pure Bile Acids by Ultraviolet Absorption Method

EXPERIMENTALS AND RESULTS

A. *Experimental Procedure*—The eluate containing each bile acid separated and extracted into a flat bottomed test tube was evaporated to dryness on a water bath. After the addition of 5 ml. of 65 per cent sulfuric acid (sp. gr. 1.56) accurately, the tubes were heated for the specified time at the specified temperature ($\pm 0.5^\circ$) in a water bath, cooled in tap water and allowed to stand for 15 minutes at room temperature. The optical density

TABLE II
Extinction Coefficient of Pure Bile Acids in Various Heating Conditions

(0.1 mg.)	28°, 60 mins.		60°, 15 mins.		60°, 60 mins.	
Cholic acid	320 m μ	0.758 S=0.0089 (10)	320 m μ	0.698 S=0.0247 (5)		
Glycocholic acid	320 m μ	0.673 S=0.0033 (10)	320 m μ	0.615 S=0.0185 (5)		
Deoxycholic acid					385 m μ	0.952
Chenodeoxycholic acid					380 m μ	1.032
Glycodeoxycholic acid			305 m μ	0.187	305 m μ	0.156
			389 m μ	0.217	389 m μ	0.420
Glycochenodeoxycholic acid			305 m μ	0.173	305 m μ	0.190
			389 m μ	0.064	389 m μ	0.126

S: standard deviation, (): number of trials.

was then determined in a Beckman model DU spectrophotometer. A standard of the corresponding pure bile acid was carried along with the sample.

B. The Extinction Coefficients of Pure Bile Acids—0.1 mg. of every pure standard solution was used. The experiment was repeated and the result is shown in Table II.

Variation in the optical density values from day to day could not be avoided. However, when duplicate samples were measured at the same time, differences between them were negligible. Therefore, in the determination of unknown amount of bile acid it is preferable to obtain the optical density of a standard simultaneously.

Pure taurine-conjugates could not be obtained. Therefore, on the assumption that spectra of taurine-conjugates were the same as those of glycine-conjugates, the extinction coefficients of taurine-conjugates were calculated from molecular weight as 90 per cent of that of glycine-conjugates.

C. Differential Determination of Conjugated Dihydroxycholanolic Acids by Ultra-violet Absorption Spectra:

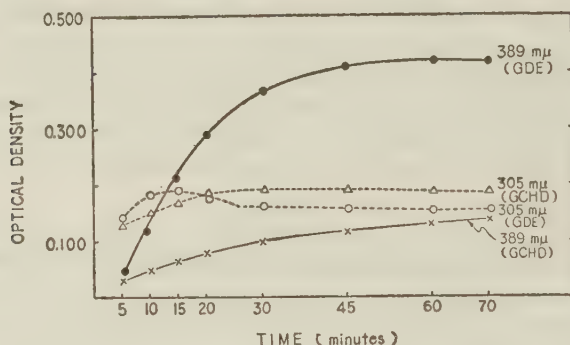


FIG. 1. Influence of heating time study on spectra of GDE and GCHD (60°).

GCHD: glycochenodeoxycholic acid { 305 mμ —△—
 { 389 mμ —x—
 GDE : glycodeoxycholic acid { 305 mμ —○—
 { 389 mμ —●—

1. *Heating Time (60°) and Variations of Absorption Spectra of Glycochenodeoxycholic Acid and Glycodeoxycholic Acid:* GCHD acid (0.1 mg.) and GDE acid (0.1 mg.) were determined at fixed time with above mentioned method. Their absorption spectra are shown in Fig. 1.

2. *Differential Determination:* GCHD acid and GDE acid could not be separated by p.p.c. with EBH-solvent. Therefore, the mixture containing both of them was determined differentially by calculation, utilizing a quality of absorption spectrum. If the optical density at 305 mμ was E (305) and the optical density at 389 mμ was E (389) in the heating condition at 60° for 60 minutes, the quantity of GDE acid (x mg.) and that of GCHD acid (y mg.) could be determined by calculation with the following expression.

a=the extinction coefficient of GDE acid at 305 m μ .
 b=the extinction coefficient of GCHD acid at 305 m μ .
 c=the extinction coefficient of GDE acid at 389 m μ .
 d=the extinction coefficient of GCHD acid at 389 m μ .

$$\begin{cases} E_{305}=0.1 (ax+by) \\ E_{389}=0.1 (cx+dy) \end{cases} \quad \begin{cases} x=0.1 \times \frac{d \cdot E_{305} - b \cdot E_{389}}{ad-bc} \\ y=0.1 \times \frac{a \cdot E_{389} - c \cdot E_{305}}{ad-bc} \end{cases}$$

The result of the test for recovery of pure bile acids is shown in Table III.

TABLE III

The Test for Recovery of Differential Determination of Conjugated Dihydroxycholic Acids by Ultraviolet Absorption

	Expected value (mg.)	Recovery	
		(mg.)	Mean (%)
GDE	0.0579	0.0617–0.0625	107 $\pm$ 1
GCHD	0.132	0.114–0.119	88.5 $\pm$ 2

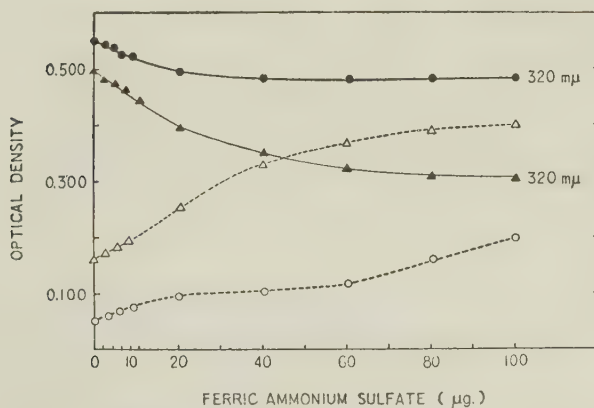


FIG. 2. Influence of ferric-ion on the spectra of free cholic acid (0.0728 mg.).

28°, 60 minutes $\begin{cases} 320 \text{ m}\mu & \text{—}\bullet\text{—} \\ 386 \text{ m}\mu & \text{---}\circ\text{---} \end{cases}$ 60°, 15 minutes $\begin{cases} 320 \text{ m}\mu & \text{---}\blacktriangle\text{---} \\ 386 \text{ m}\mu & \text{---}\triangle\text{---} \end{cases}$

D. *The Influence of Ferric-ion*—Aliquot quantity of ferric ammonium sulfate was added to C acid (0.0728 mg.). Variations of absorption spectra in the heating condition at 60° for 15 minutes and at 28° for 60 minutes are shown in Fig 2. In the heating condition at 60° for 15 minutes, as the

TABLE IV
Influence of "dirt" in the Filter Paper on the Spectrum of Glycolic Acid

GC (0.104 mg.)			28°, 60 minutes				60°, 15 minutes			
			E (Gc-BI)			E-BI	E (Gc-BI)			E-BI
			320m μ	Recovery (%)	386 m μ		320 m μ	Recovery (%)	386 m μ	
*	**	***								
			0.673	(100)	0.067		0.607	(100)	0.167	
No. 51	unwashed	+	0.535	(79.5)	0.081	0.059	0.495	(81.5)	0.208	0.060
No. 51	washed	+	0.592	(88)	0.090	0.030	0.495	(81.5)	0.210	0.020
No. 53	washed	+	0.628	(93.5)	0.090	0.037	0.553	(91.0)	0.243	0.035

* The kind of the filter paper.

** Washing procedure with ethanol.

*** p.p.c. by EBH-solvent.

concentration of ferric-ion increased, the optical density at $320\text{ m}\mu$ decreased remarkably, while the optical density at $386\text{ m}\mu$ increased remarkably. In heating condition at 28° for 60 minutes, decrease of the optical density at $320\text{ m}\mu$ was not so remarkable. Absorption spectra of free and conjugated DIH acids were not influenced at all by ferric-ion.

E. Influence of Types and Washing of the Filter Paper—The filter paper was washed with ethanol for three days, subjected to p. p. c., cut into pieces ($3.5 \times 12\text{ cm.}$) and extracted.

A definite volume of bile acid was added to eluate and the optical density was measured. The elute which was not added to bile acid was used as a "blank".

The result using GC acid is shown in Table IV. It was proved that C acid gave the same result as when using GC acid, and the spectra of free and conjugated DIH acids were not influenced at all by "dirt".

F. The Test for Recovery of Pure Bile Acids by This Method—Filter paper No. 53 was used. A definite volume of pure bile acid was spotted and subjected to p. p. c. The result of the test for recovery is shown in Table V. By preliminary experiment, it was found that extraction for 24 hours was enough.

TABLE V
Recovery of Pure Bile Acids after Paperchromatography

Solvent of p. p. c.	Bile acid	Added amount (mg.)	Number of trials	Mean recovery (per cent)	Range (per cent)
EBH-Solvent	GDE	0.145	4	98.4	96.4–102.2
	GC	0.104	5	93.4	92.1– 95.0
TEW-Solvent	DE	0.064	5	98.6	96.2–103.2
	C	0.182	5	93.2	91.8– 95.4

DISCUSSION

A. Optimum Heating Conditions in the Determination of Free and Conjugated Cholic Acids—In the quantitative determination performed by Mosbach *et al.* (14), Inutsuka (11) and Eriksson and Sjövall (15), free and conjugated C acids were heated at 60° for 15 minutes, and in the determination performed by Kier (13) and Kazuno (16), they were allowed to stand at room temperature for 50 minutes. In each case, the acids were determined by measuring the optical density at the absorption maximum, $320\text{ m}\mu$. After a comparative study on these two heating conditions, the heating condition of 28° for a 60 minute period was adopted in this paper, because it had small standard deviation (Table III) and was hardly affected by ferric-ion (Fig. 2) or "dirt" in the filter paper (Table IV). In the present experiment, the maxima for the spectra of free and conjugated C

acids were found at 320 $m\mu$ and 386 $m\mu$, not at 320 $m\mu$ and 389 $m\mu$ as in the reports published earlier.

B. Differential Determination of Conjugated Dihydroxycholanic Acids—Conjugated DIH acids in human bile obtained conjugated-CHD acid and conjugated DE acid. After heating them at 60° for 15 minutes, Eriksson and Sjövall (15) measured the optical density of conjugated CHD acid at 305 $m\mu$ and that of conjugated-DE acid at 389 $m\mu$ and determined them. When conjugated CHD acid and conjugated DE acid could not be separated by p.p.c., they determined their total amount, considering that in the heating condition at 50° for 15 minutes their extinction coefficients at 305 $m\mu$ were the same (17).

The author determined GCHD acid and GDE acid by calculation, utilizing the fact that the absorption maximum of GCHD acid at 305 $m\mu$ was larger than that of GDE acid, and on the other hand, absorption maximum of GDE acid at 389 $m\mu$ was larger than that of GCHD acid.

With this calculation, the calculated value of GDE acid was larger than the expected value as 107 ± 1 per cent, and the calculated value of GCHD acid was smaller than the expected value as 88.5 ± 2 per cent. The optical density at 305 $m\mu$ usually became smaller than the expected value by unknown factors, when both of these pure bile acids were mixed.

C. Influence of Kinds and Washing of the Filter Paper—"Dirt" in the filter paper reduced the optical density of free and conjugated C acids at 320 $m\mu$ and it also reduced the recovery. To remove "dirt", the filter paper was washed with ethanol for three days. As a result of washing, the "blank" reading decreased but the recovery test did not increase so much. Therefore, the filter paper No. 53 was used. In this case, in spite of increase of "blank", the recovery of free and conjugated C acids obtained a remarkably good result. Therefore, ferric-ion in "dirt" was supposed to interfere with the absorption spectra of free and conjugated C acids. It was quite difficult to remove this ferric-ion by washing with only ethanol for three days, so it was preferable to use the filter paper No. 53, which contained less ferric-ion and ashes. The absorption spectra of free and conjugated DIH acids were not influenced at all by "dirt" in the filter paper.

III. Results of the Determination of Bile Acids in Human Gallbladder Bile by This Method and the Test for Recovery of Pure Bile Acids Added to Human Bile

EXPERIMENTALS AND DISCUSSION

A. The Test for Recovery of Pure Free Bile Acids added to Human Bile—Free DE acid and C acid were added to "the crude bile sample solution" of a patient with peptic ulcer, which did not contain free bile acid. Recovery test was performed five times. The results are shown in Table VI.

B. Results of the Determination of Bile Acids in Human Gallbladder Bile by this Method—These results are shown in Table VII. In the bladder bile

TABLE VI
Recovery of Bile Acids Added to Human Bile

	Added amount (mg.)	Number of trials	Mean recovery (per cent)	Range (per cent)
DE	0.0604	5	97.1	95.4-98.4
C	0.0728	5	90.7	88.6-92.6

Cholic acid (0.728 mg./ml.) and deoxycholic acid (0.604 mg./ml.) were added to the bile sample solution of the patient with peptic ulcer (H.H.). 0.1 ml. of the bile sample solution was spotted.

TABLE VII
Result of the Determination of Bile Acids in Human Gallbladder Bile

		Glycine-conjugated bile acid (mg./ml.)			Taurine-conjugated bile acid (mg./ml.)			Free bile acid (mg./ml.)	
		GC	GCHD	GDE	TC	TGHD	TDE	C	DIH
T. T.	Cholecystitis with gall stone	2.80	6.28	1.90	0.80	1.46	0.75	0.09	0.34
S. Y.	„	1.00	0.02	2.01	2.68	1.03	1.32	0.01	0.34
I. M.	„	0.13	0.15	0.03	0.29	0.17	0.01	0	0
J. O.	„	1.33	1.90	0.05	1.76	1.41	0.32	0	0
K. S.	Cholecystitis without gall stone	3.00	9.45	0	0.46	1.43	0	0	0
S. M.	Peptic ulcer	11.2	35.0	4.15	3.00	8.15	4.75	0	0
H. H.	„	21.2	39.0	4.82	13.0	2.18	1.64	0	0

G: glyco-, T: tauro-, C: cholic acid, CHD: chenodeoxycholic acid, DIH: dihydroxycholanolic acids.

of a patient with cholecystitis with gall stone, free bile acids were found and conjugated bile acids decreased remarkably. On the contrary, in bladder bile of the patient except cholecystitis with gall stone, free bile acid could not be found.

That this quantitative and qualitative change (the appearance of free bile acids) of bile acid in bile had some significance on the genesis of gall stones was stated by Miyake (18, 19).

Previously published reports (20) stated that in Japanese bile, there were no taurine-conjugates, but this study detected considerable quantities of these two taurine-conjugates in all examples.

For the differential determination of free bile acids by this method, it was necessary to spot 5-10 volumes of the quantity for the determination of conjugates on the starting spots. Free CHD and DE acid, which could not be separated by p. p. c., were determined together as free DIH acids.

SUMMARY

1. A method for microdifferential determination of bile acids by use of p. p. c. and ultraviolet absorption was devised. With this method, free, glycine-conjugated and taurine-conjugated bile acids were determined quantitatively without any interference by lipids.

2. To remove the lipids in bile which interfere with the determination, two-step-p. p. c. with 50 per cent methanol (pH 12) or an extraction procedure with 50 per cent ethanol and petroleum ether was performed and after this treatment, p. p. c. was allowed to proceed.

3. Taurine-conjugates were separated by p. p. c. with a new solvent containing ethyl butyrate (or isoamyl formate)/heptane/70 per cent acetic acid.

4. After heating at 60° for 60 minutes, conjugated chenodeoxycholic acid and conjugated deoxycholic acid which could not be separated by p. p. c. were determined differentially by utilizing a quality of absorption spectra.

5. The optimum heating conditions for free and conjugated cholic acids were investigated and it was found that the heating condition of 28° for 60 minutes gave more precise results than that of 60° for 15 minutes.

6. The maxima for the spectra of free and conjugated cholic acid, were found at 320 m μ and 386 m μ , not at 320 m μ and 389 m μ as in previously published reports.

7. As determinations of free and conjugated cholic acid by p. p. c. were much influenced by ferric-ion in the filter paper, Toyo filter paper No. 53 which contained less ashes and ferric-ion was preferable.

8. Recoveries of pure bile acids by this method were as follows: Deoxycholic acid (98.6 per cent), cholic acid (93.2 per cent), glycodeoxycholic acid (98.4 per cent), and Glycocholic acid (93.4 per cent).

6. Recoveries of bile acid added to human bile were as follows: Deoxycholic acid (97.1 per cent), and cholic acid (90.7 per cent).

10. The reports published before said that in Jananese bile there were no taurine-conjugates, but as a result of this experiment, taurine-conjugates were found in all examples.

11. In bladder bile of some cases of cholecystitis with gall stone, small amounts of free bile acids were also detected.

The author wished to thank Prof. H. Miyake, Faculty of Medicine, Kyushu University, for his interests in this work and many valuable instructions. Thanks are also due to Prof. Y. Oshima and Assistant Prof. M. Funatsu, Faculty of Agriculture, Kyushu University, for their valuable discussions.

REFERENCES

- (1) Sjövall, J., *Acta Chem. Scand.*, **6**, 1552 (1952)
- (2) Beyreder, J., and Rettenbacher-Daubner, H., *Monatsh.*, **84**, 99 (1953)
- (3) Kritschewsky, D., and Kirk, M. R., *J. Am. Chem. Soc.*, **74**, 1413 (1952)
- (4) Tanaka, K., and Takeda, K., *J. Biochem.*, **39**, 333 (1952)
- (5) Sjövall, J., *Acta Chem. Scand.*, **8**, 339 (1954)
- (6) Kuroda, M., *J. Japan. Biochem. Soc.*, **27**, 301 (1955)
- (7) Sjövall, J., *Arkiv Kemi*, **8**, 299 (1955)
- (8) Sjövall, J., *Arkiv Kemi*, **8**, 317 (1955)
- (9) Sjövall, J., *Acta Chem. Scand.*, **9**, 1034 (1955)
- (10) Sjövall, J., *Acta Chem. Scand.*, **10**, 1051 (1956)
- (11) Inutsuka, T., *Fukuoka Acta Medica*, **48**, 733 (1957)
- (12) Bandow, F., *Biochem. Z.*, **301**, 37 (1939)
- (13) Kier, L., *J. Lab. Clin. Med.*, **40**, 755 (1952)
- (14) Mosbach, E. H., Kalinsky, H. J., Halpern, E., and Kendall, F. E., *Arch. Biochem. Biophys.*, **51**, 402 (1954)
- (15) Eriksson, S., and Sjövall, J., *Arkiv Kemi*, **8**, 303, 311 (1955)
- (16) Kazuno, T., Seno, H., Goto, Y., and Fuziwara, K., *J. Japan. Biochem. Soc.*, **23**, 133, (1956)
- (17) Sjövall, J., *Acta Chem. Scand.*, **10**, 1051 (1956)
- (18) Miyake, H., and Murakami, E., *Medicine*, **11**, 98 (1954)
- (19) Miyake, H., and Honda, A., *J. J. S. S.*, **48**, 165 (1947)
- (20) Yamaguchi, S., *J. Japan. Biochem. Soc.*, **23**, 1125 (1951)

THE STRUCTURE OF GLYCOPEPTIDES OBTAINED FROM TAKA-AMYLASE A

By AKIRA TSUGITA AND SHIRO AKABORI

(From the Laboratory of Biochemistry, Faculty of Science,
Osaka University, Osaka)

(Received for publication, September 5, 1958)

Akabori *et al.* (1) and Hanrahan and Caldwell (2) independently reported that Taka-amylase A (TAA) gives a positive Molisch reaction. Hayashi (3) also obtained evidence indicating the presence of carbohydrate components in TAA. Recently Hanafusa, Ikenaka and Akabori (4) have finally found 8 moles of mannose, 1 mole of xylose and 2 moles of hexosamine in crystalline TAA.

The present paper deals with the isolation of sugar peptides from partial hydrolysates of TAA and their characterization. It has thus been confirmed that these carbohydrate components are actually the integral constituents of the enzyme molecule. It has been suggested that the sugar are contained in TAA in the following combination:

-(mannose 8-9, xylose)-O-seryl-glutamyl-aspartyl-glycyl-(alanine, threonine)-.

MATERIALS AND METHODS

Materials—TAA was purified from "Taka-Diastase" (Sankyo) and recrystallized three times according to the preceding papers (5, 6).

A protease prepared from *Streptomyces griseus* was used (7) for enzymatic degradation experiments.

Paper Chromatography—a) For the identification of carbohydrate components and amino acids, one-dimensional chromatography was employed using the solvent systems composed of *n*-butyl alcohol: acetic acid: water (4:1:1 by volume) and pyridine: acetic acid: water (6:2:3 by volume). b) For the estimation of dinitrophenyl- (DNP-) amino acids, two-dimensional chromatography was used according to Levy's (8) and Koch's (9) methods. The solvent systems used were *n*-butyl alcohol saturated with 0.1 per cent aqueous ammonia and 1.5 *M* phosphate buffer of pH 6.0. c) For the separation and the estimation of sugar peptides and their DNP-derivatives, especially for the estimation of their purity, two-dimensional paper chromatography was employed using the solvent systems described above. One-dimensional chromatography was applied to the separation and purification of peptides using acetic acid-butyl alcohol-water. The phosphate buffer was used as a developing solvent in case of the DNP-derivatives of the sugar peptides.

Ion Exchange Column Chromatography—For the isolation of glyco-peptides from other peptides, the ion exchange column technique was applied. A sulfonated polystyrene cation exchanger, Dowex 50 (-X4), 200-400 mesh, was employed. The conditions for elution and the elution pattern are recorded in Fig. 1. The amino groups and the total amount of carbohydrate components were analysed by the ninhydrin method (10) and a modification

of the orcin-sulfuric acid method (11) respectively.

Determination of Amino-Terminal Amino Acid Residues—Sanger's dinitrophenylation method (12) was applied to the determination of amino-terminal amino acid in the peptides.

Determination of Carboxyl-Terminal Amino Acid Residues—The hydrazinolysis procedure of Akabori and Ohno (16) was used for the carboxyl-terminal analysis of peptides.

Determination of Amino Acid Composition—The amino acid analysis was conducted by two methods: a) After dinitrophenylation Levy's method of two-dimensional paper chromatography (8) was applied. b) When more precise analysis was required, cation column chromatography was employed according to Moore and Stein's method (13).

Quantitative Analysis of Hexoses and Hexosamines—A modification of the orcin-sulfuric acid procedure was applied to determine the total hexose. To a sample solution (0.5 ml. or 1.0 ml.) which contained 5–60 γ of hexose were added 1 ml. of 1 per cent orcin solution in 30 per cent sulfuric acid and then 4 ml. of 75 per cent sulfuric acid under vigorous stirring. The solution was kept in a boiling water bath for 10 minutes. The developed colour was determined spectrophotometrically at 530 $m\mu$.

Determination of hexosamine was carried out by Elson-Morgan's method (14).

RESULTS AND DISCUSSIONS

Preparation and Purification of Glycopeptides—300 mg. of TAA was digested with 6 mg. of *Streptomyces* protease for two days at 37°, the solution being

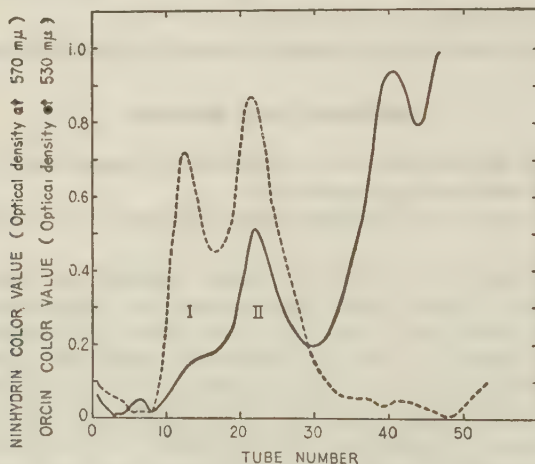


FIG. 1. Ion Exchange chromatogram of sugar peptides in Taka-amylase A.

----- concentration of sugar, — concentration of amino-residue.

Dowex 50 $\times$ 4 (ϕ 20 $\times$ 800 mm.) 0.2 *N* citrate buffer pH 2.2.

maintained at pH 7.5–8.0. The resulting peptide mixture was chromatographed on a cation exchanger (Dowex 50 $\times$ 4) column at pH 2.2, as shown in Fig. 1. Two sugar-containing fractions (Fr. I and Fr. II) were obtained in the early stage of elution, where no amino acids could be

eluted. It was important to perform the elution procedure as rapidly as possible, since otherwise more than two sugar-containing fractions appeared. This probably due to the hydrolytic cleavage of sugar peptides by the action of the resine.

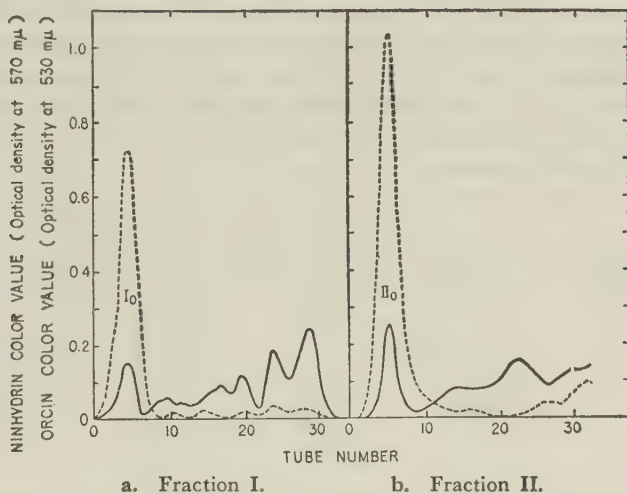


FIG. 2. Rechromatogram of sugar peptide.

Dowex 50×4 (ϕ 10×300 mm.), elution by H_2O .

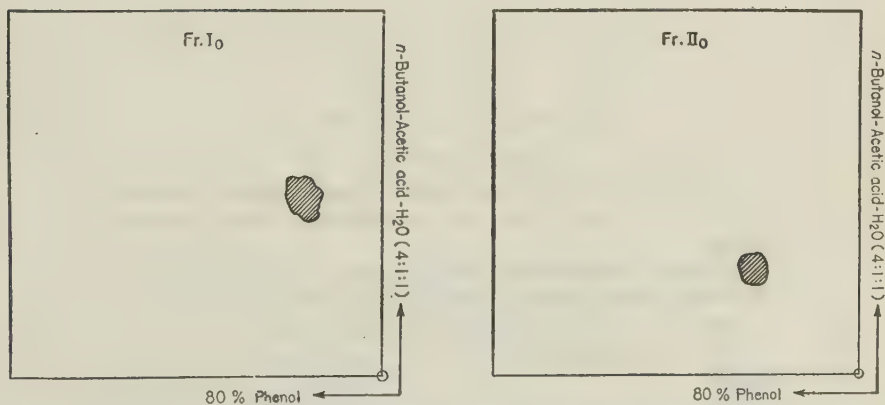


FIG. 3. Estimation of the purity of sugar peptide fractions in Taka-amylase A by paper chromatography. (Filter paper: Toyo-Roshi No. 51)

In any event, these two sugar-containing fractions were collected and freeze-dried, and subjected to a second ion exchange chromatography (Dowex 50-×4, H form, 200-400 mesh, elution with water) to further remove impurities such as free peptides, amino acids and inorganic cations.

Figs. 2a and 2b show the results of such rechromatography. After these purification procedures, the glycopeptides, Fr. I₀ and Fr. II₀ seemed to be sufficiently pure. Their purity was estimated by two-dimensional paper chromatography, in which the ninhydrin positive and α -naphthol positive spots coincided and no other spots were detected, as shown in Fig. 3.

Zone electrophoresis using a vertical starch column was carried out to further confirm the purity of the sugar peptides. The condition used were as follows: column dimension, 1.2×40 cm.; buffer solution, pyridine-acetate buffer pH 6.5; voltage gradient, 10–15 volts/cm.; and time, 10–20 hours. One of the diagrams, which is shown in Fig. 4, also indicates that the fraction is practically pure.

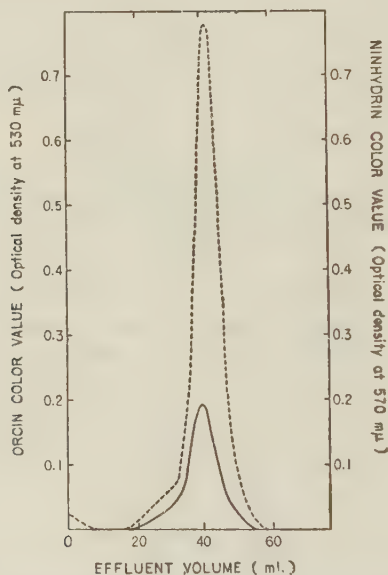


FIG. 4. Estimation of the purity of sugar peptide fraction (Fr. II) in Taka-amylase A by a vertical starch column electrophoresis.

Column : potato-starch : hyflo supercel (4 : 1 *w/w*) 12×300 mm.

Buffer : pH 6.5 pyridine-acetic acid-H₂O (80 : 3 : 917).

300 volt, 65 mA. 14 hours, at room temperature.

— concentration of sugar, ---- concentration of amino residue.

Determination of the Composition of Glyco Peptides—The purified glycopeptides were separately hydrolysed with 2 *N* hydrochloric acid for about 2 hours at 100° and the hydrolysate evaporated at temperatures below 40° *in vacuo*. Under these conditions, only the glycosidic linkages of the glycopeptides were hydrolysed leaving almost all the peptide bonds untouched. The hydrolysates were passed through Dowex 50-×8 (H form) column and repeatedly washed with water to remove the the liberated sugars. The

adsorbed peptide fractions were eluted with 5 *N* aqueous ammonia. The eluates were evaporated and subjected to hydrolysis with 6 *N* hydrochloric acid for 16 hours at 100°. This resin treatment was necessary to avoid an unfavorable browning reaction between the amino acids and the sugar.

The sugars which passed through the column were identified by paper chromatography (Fig. 5). The each spot on the chromatograms was eluted with water and analysed by the orcin-sulfuric acid or orcin-hydrochloric acid method (15). It was shown that a comparatively good agreement exists between the carbohydrate compositions of both Fr. I₀ and Fr. II₀, both of them were shown to contain mannose and xylose in a molar ratio of 7-8:1 and that, on a molar basis, the total amount of carbohydrates was about ten times as much as that of the amino terminal residue in peptides as determined by the ninhydrin method.

It is interesting to note that the isolated glycopeptides contain all the mannose and xylose molecules originally present in TAA itself. The amino

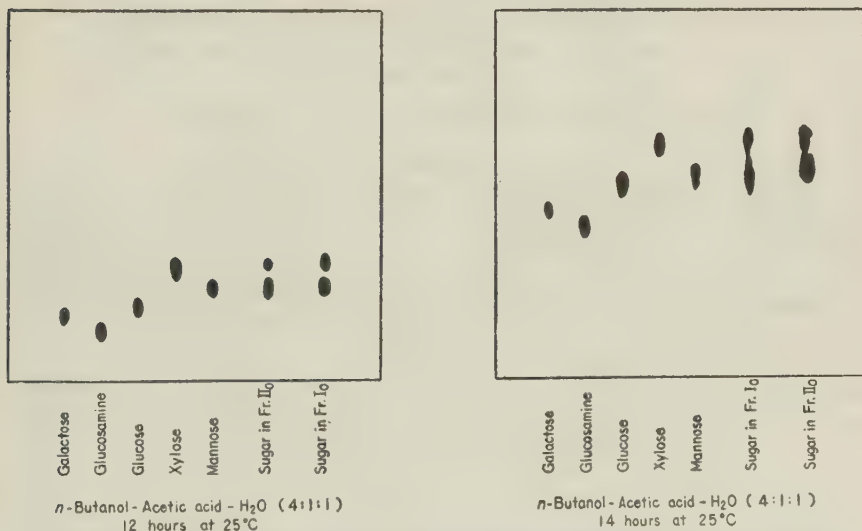


FIG. 5. Paper chromatogram of carbohydrate component in sugar peptides.
(Filter paper: Toyo-Roshi No. 51)

acid compositions of the carbohydrate-free fractions were determined by one-dimensional paper chromatography after hydrolysis and dinitrophenylation according to Levy (8,9) and by column chromatography of Moore and Stein (13). Figs. 6a and 6b show the elution curves obtained in the column chromatography of Fr. I₀ and Fr. II₀, respectively. The amino acid and carbohydrate compositions of the glycopeptides are recorded in Table I. It may be seen that Fr. I₀ contained 7-8 moles of mannose, 1 mole of xylose, and each 1 mole of serine, aspartic acid, glutamic acid. Fr. II₀ contained, in addition to the components found in Fr. I₀ one mole

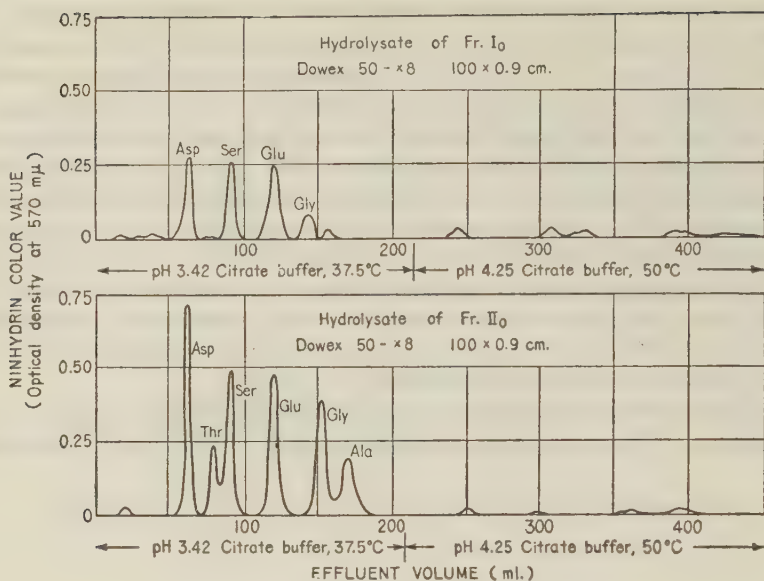


FIG. 6. Column chromatogram of amino acid composition.

TABLE I

Amino Acid and Carbohydrate Component of Sugar Peptides

Fr. No.	Fr. I ₀		Fr. II ₀	
Method	Moore-Stein's ²⁾ (Dowex 50-X8)	Levy's ³⁾ (DNP)	Moore-Stein's ²⁾ (Dowex 50-X8)	Levy's ³⁾ (DNP)
Ser ¹⁾	1.000	1.00	1.000	1.00
Asp	1.009	} 2.47 ⁴⁾	1.217	} 2.26 ⁴⁾
Glu	1.219		1.164	
Gly	0.215 (?)	—	1.145	1.07
Ala	—	—	0.533	0.67
Thr	—	—	0.312	0.52
Mannose	8.11 ⁵⁾		7.89	
Xylose	1.13		0.95	
Glucosamine	0.11		0.06	
Total sugar	10.3 ⁶⁾		9.2	

1) Quantities of amino acids were expressed as the relative mole-ratio to serine.

2) These values were corrected by the column recovery and the decomposition factor of hydrolysis.

3) These values were also corrected according to Levy's report.

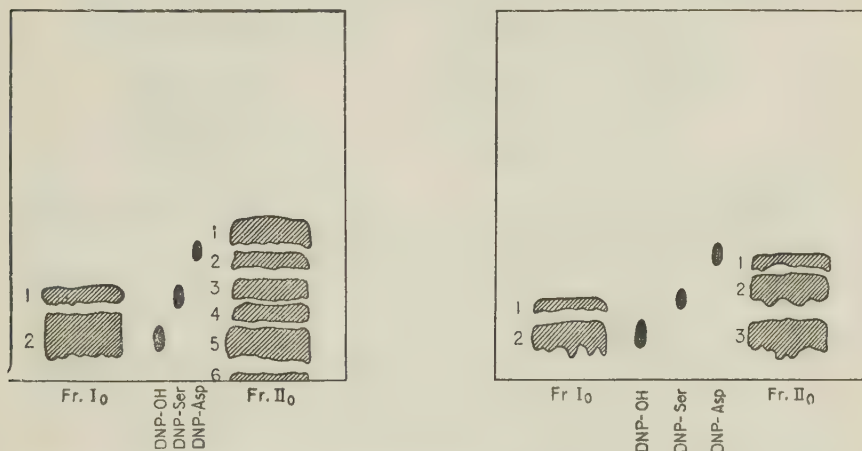
4) DNP-Asp and DNP-Glu could not be separated in this experiment.

5) Quantities of carbohydrate component were expressed as the mole-ratio to amino residue of the peptide.

6) Total sugar was determined by orcin reaction.

of glycine, 0.6 mole of alanine and 0.4 mole of threonine. Hexosamine could not be detected in either of the sugar peptides.

Structure of Glycopeptides—The glycopeptides were very difficult to be converted into dinitrophenyl derivatives under the usual dinitrophenylation conditions, probably due to the steric hindrance by the sugar residues. However, when the reaction was conducted at pH 10.5, the peptides were found to be convertible to dinitrophenyl derivatives. But the formed dinitrophenyl derivatives seemed to have lost a part of sugar components during the reaction. After purification of the dinitrophenyl derivatives by paper chromatography, they were hydrolysed and their amino terminal amino acids were determined. Thus the amino terminal residues of both Fr. I₀ and Fr. II₀ were identified as serine (Fig. 7b and Table II).



a) DNP-derivatives of partial hydrolysates from sugar peptides.

b) DNP-derivatives of sugar peptides.

FIG. 7. Paper chromatogram of DNP-peptides
(1.5 M Phosphate buffer pH 6.5)

The fact that serine is the actual amino terminal residue of the glycopeptides received further support by a following experiment in which these peptides were partially hydrolysed to split the sugar-peptides linkage and the amino termini of the resulting sugar-free peptides were investigated.

The carboxyl termini of the glycopeptides were determined by the hydrazinolysis method (16). The carboxyl terminal residue of Fr. I₀ was identified as aspartic acid and that of Fr. II₀ as threonine although a small quantity of alanine was also detected in the latter case.

To determine the amino acids sequence of these peptides, they were partially hydrolysed with 2 N hydrochloric acid for about 3 hours at 100°, and the hydrolysates were dinitrophenylated and fractionated by one-dimensional paper chromatography as shown in Fig. 7a.

The dinitrophenylated peptides thus fractionated were then completely hydrolysed and the hydrolysate were analysed both for the amino terminal residues and for the amino acid composition by the usual methods. The dinitrophenylated peptides obtained by the dinitrophenylation at pH 10.5 were also analysed in the same manner. The results of these experiments are summarized in Table II.

TABLE II
Structure of DNP-Derivatives from Modified Sugar Peptide

Fraction No.	N-terminal (DNP-)	Composed amino acid	Peptide sequence (DNP-)
DNP-derivatives of partial hydrolysate of sugar peptide			
I-1	Ser	Glu, Asp	Ser-(Glu, Asp)
I-2	DNP-OH ¹⁾	—	—
II-1	Ser	Glu, Asp	Ser-(Glu, Asp)
II-2	Acidic ²⁾	—	Asp or Glu
II-3	Acidic	Asp, Gly	Glu-(Asp, Gly)
II-4	DNP-OH	—	—
II-5	Gly, Ser		Gly or Ser
DNP-derivatives of sugar peptide at pH 10.5			
I-1	(Ser)	Glu, Asp	Ser-(Glu, Asp)
I-2	DNP-OH	—	—
II-1	Acidic	Asp, Gly, Ala, Thr	Glu-(Asp, Gly, Ala, Thr)
II-2	Ser	Glu, Asp, Gly, Ala, Thr	Ser-(Glu, Asp, Gly, Ala, Thr)

1) DNP-OH=dinitrophenol.

2) 'Acidic' means acidic amino acids, and whether aspartic acid or glutamic acid was not determined.

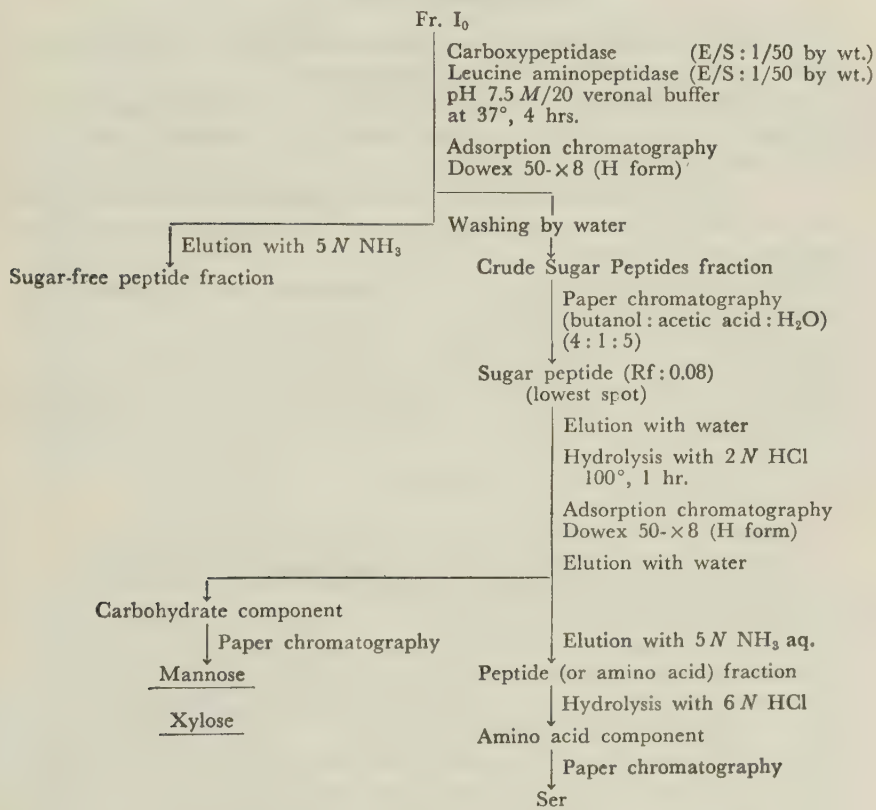
Based on these results, the structure of the peptide in Fr. I₀ was suggested to be, seryl-glutamyl-aspartic acid, and that in Fr. II₀ to be, seryl-glutamyl-aspartyl-glycyl-(alanine, threonine).

In order to elucidate the mode of linkage of the sugar components to the peptide moiety of the glycopeptides, Fr. I₀ was subjected to the combined action of pancreatic carboxypeptidase A (17) and leucine aminopeptidase (18) at 37° for 4 days. The enzyme were occasionally added to the reaction mixture to reinforce the enzymatic activities. The resulting digest was treated with a Dowex 50-×8 (H form) column and orcinol-positive fractions were collected. These fractions were then chromatographed on paper using a solvent system composed of butyl alcohol, acetic acid and water (4:1:5). Since only one spot having the lowest *R_f* value was found to be positive in both the ninhydrin and α -naphthol reactions, this spot was eluted from the paper and the eluate was hydrolysed with 2 *N* hydrochloric acid for 1 hour

at 100°. The sugars liberated in this procedure were separated by the treatment with a Dowex 50 (H-form) column, and the fractions containing amino acids (or peptides) were further hydrolysed with 6 *N* hydrochloric acid for 5 to 10 hours at 100°. On paper chromatography of this hydrolysate, it was found that only serine was present. It was also found that the carbohydrate fraction separated above was composed of mannose and xylose. The procedures employed in these experiments are summarized in Scheme 1.

SCHEME 1

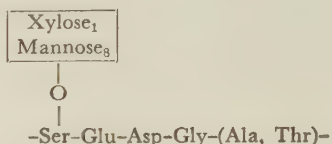
*Determination Procedure of the Glycosidic Linkage in the Sugar Peptide
Obtained from Taka-amylase A*



From the results just described, it is suggested that all the sugar components in the isolated glycopeptides are linked to the peptide moiety through the serine residue. It is also possible that the hydroxyl group of serine is responsible for the sugar linkage, since the sugar-serine compound was positive to ninhydrin as well as to orcinol. This consideration is coincided with that the sugar peptides could not be dinitrophenylated in the ordinary reaction conditions but when pH was raised to 10.5, they could

be dinitrophenylated. The nature of hexosamines originally present in the TAA molecule is still to be elucidated.

Summarizing all the data presented in this paper, it may be concluded that the following structure exists in TAA:



SUMMARY

1. It was confirmed that the carbohydrates found in Taka-amylase A constitute an integral part of the enzyme molecule.

2. All the carbohydrate components except for the hexosamines seem to be linked to the enzyme proteins through the hydroxyl group of a particular serine residue.

3. In the glycopeptides, isolated from the partial hydrolysates of Taka-amylase A, one mole of xylose and 7-8 moles of mannose are present per one amino terminal residue.

4. The amino acid sequence of the glycopeptide was suggested to be seryl-glutamyl-aspartyl-glycyl-(alanine, threonine).

The authors wish to express their gratitudes to Sankyo Co., Ltd. for their kind supply of 'Takadiastase Sankyo' and to Dr. T. Ikenaka for his valuable advice during the course of this research.

REFERENCES

- (1) Akabori, S., Ikenaka, T., and Hagihara, B., *J. Biochem.*, **41**, 581 (1954)
- (2) Hanrahan, V.M., and Caldwell, M.L., *J. Am. Chem. Soc.*, **75**, 4030 (1953)
- (3) Hayashi, S., *J. Chem. Soc. Japan*, **62**, 1 (1941)
- (4) Hanafusa, H., Ikenaka, T., and Akabori, S., *J. Biochem.*, **42**, 55 (1955)
- (5) Akabori, S., Hagihara, B., and Ikenaka, T., *Proc. Japan Acad.*, **27**, 350 (1951)
- (6) Akabori, S., Ikenaka, T., and Hagihara, B., *J. Biochem.*, **41**, 577 (1954)
- (7) Nomoto, M., and Narahashi, K., *Symposia on Enzyme Chem.*, **12**, 24 (1957)
- (8) Levy, A.L., *Nature*, **174**, 126 (1954)
- (9) Koch, G., and Weidel, W., *Z. physiol. Chem.*, **303**, 213 (1956)
- (10) Moore, S., and Stein, W.H., *J. Biol. Chem.*, **176**, 367 (1948)
- (11) Sørensen, M., and Haugaard, G., *Biochem. Z.*, **260**, 247 (1933)
- (12) Sanger, F., *Biochem. J.*, **39**, 507 (1954)
- (13) Moore, S., and Stein, W.H., *J. Biol. Chem.*, **192**, 663 (1951)
- (14) Elson, L.A., and Morgan, W.T.J., *Biochem. J.*, **27**, 1824 (1933)
- (15) Albaum, H.G., and Umbreit, W.W., *J. Biol. Chem.*, **167**, 369 (1947)
- (16) Akabori, S., Ohno, K., Ikenaka, T., Okada, Y., Hanafusa, H., Tsugita, A., Haruna, I., Sugae, K., and Matsushima, T., *Bull. Chem. Soc. Japan*, **29**, 507 (1956)
- (17) Anson, M.L., *J. Gen. Physiol.*, **20**, 663 (1937)
- (18) Smith, E.L., and Slonim, N.B., *J. Biol. Chem.*, **176**, 835 (1948)

EFFECT OF FATTY ACIDS ON THE PROTEOLYSIS OF PROTEINS

II. EFFECT OF C_8 - C_{14} FATTY ACIDS ON THE TRYPTIC DIGESTION OF SERUM ALBUMIN

By MICHIO KONDO

(From the Laboratory of Enzymology, Institute of Applied Microbiology,
University of Tokyo, Tokyo)

(Received for publication, September 24, 1958)

In the previous paper (1) it was shown that ovalbumin became susceptible to tryptic digestion by the addition of fatty acids, nearly as much as the heat denatured protein. The per cent digestion increased with an increase of the carbon chain of fatty acids within a range from 8 to 14.

The laevo rotation of an ovalbumin solution also increases upon the addition of fatty acids. These results suggest that the fatty acids cause a modification of the protein molecule identical with or very similar to heat denaturation. On the other hand, Boyer *et al.* (2) reported that the stability of serum albumin against heat denaturation measured by physico-chemical methods was increased by fatty acid. Their results seem to be inconsistent with results of the author on the effect of fatty acids on the digestion of ovalbumin. Accordingly, in the present paper the effects of added fatty acids on the susceptibility to tryptic digestion of serum albumin are studied.

MATERIALS AND METHODS

(A) *Materials*—*Crystalline Serum Albumin*: This material was prepared according to Kondo (3). *Crystalline trypsin*—The enzyme was kindly supplied from Mochida Seiyaku Co., and 1 mg of this material had a specific activity of 1,270 units.

Fatty Acids: Fatty acids used were *n*-caprylic, *n*-capric, *n*-lauric and *n*-myristic acids. All the acids were kindly supplied from Prof. Funahashi's Laboratory.

(B) *Methods*—Two sets of 5 ml. of 1 per cent serum albumin solution which contained a definite amount of fatty acid and 0.45 mM of phosphate buffer pH 7.8, were treated as follows:

a. no heat treatment.

b. heated at 100° for 10 minutes.

Each of them was placed into a 20 ml. test tube and kept in a water bath at 37°. Then, 1 ml. of 0.5 per cent trypsin solution was added to the test tube and mixed. At different time intervals, namely, after 1, 5, 10, 20 and 40 minute incubation, 1 ml. of the reaction mixture was taken out and poured into 10 ml. of 5 per cent trichloroacetic acid solution and the resulted precipitate was filtered off.

The optical density of the filtrate was determined with a spectrophotometer at 280 $m\mu$, and the percent digestion (TCA soluble "tyrosine"/total "tyrosine" of serum albumin

$\times 100$) was calculated. As a blank test, 10 ml. of the trichloroacetic acid solution were added to 0.83 ml. of the substrate solution and 0.17 ml. of the enzyme solution. The absorbance of this filtrate was subtracted from the above determined absorbances.

The specific rotation of Serum albumin was determined as follows. The albumin solution which contained fatty acid was incubated for 20 minutes at 37° , followed by centrifugation (12,000 r.p.m., 10 minutes) to remove the resulting turbidity. No detectable loss of nitrogen compounds by centrifugation was observed. The specific rotation was determined in a 5-cm. tube with a Lippich's polarimeter at 25° .

RESULTS

1. *Digestion of Serum Albumin after Heat-Treatment*—One per cent solution of serum albumin was heated at different temperatures for 10 minutes. The resulting solutions were digested by trypsin. The time course of the digestion is shown in Fig. 1. In contrast to the previous observation on ovalbumin, the highest digestibility of serum albumin was obtained after heat treatment at 100° . The intact protein was digested by trypsin much faster than native

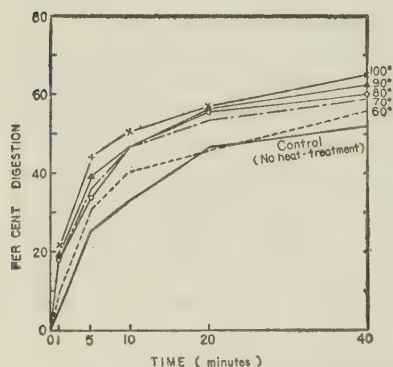


FIG. 1. Digestion of heat-treated serum albumin by trypsin.

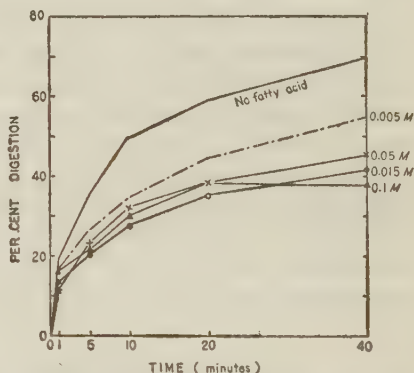


FIG. 2. Effect of C_8 fatty acid added on the digestion of serum albumin by trypsin.

ovalbumin. This is not likely due to the denaturation of the substrate, since both the commercial crystalline serum albumin preparation obtained from The Armour Laboratories Chicago, Illinois* and a fresh horse serum were digested at the same rate by trypsin.

2. *Effect of Added Fatty Acid on the Tryptic Digestion of Serum Albumin*—Generally speaking, addition of fatty acids, from C_8 to C_{14} at concentrations ranging from 0.0025 M to 0.1 M to serum albumin resulted in a decrease of digestibility by trypsin as shown in Figs. 2, 3, 4 and 5. This is in contrast with the previous observation on ovalbumin where the fatty acid addition stimulated the digestion by trypsin. The lowest digestion of serum albumin during 20 minutes was observed at 0.015 M , 0.005 M , 0.0025 M and 0.0025 M for C_8 , C_{10} , C_{12} and C_{14} acids, respectively.

* This was kindly supplied from Dr. Terayama.

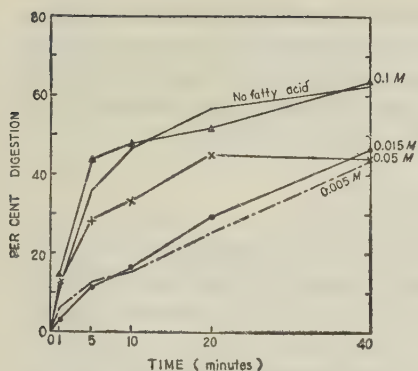


FIG. 3. Effect of C₁₀ fatty acid added on the digestion of serum albumin by trypsin.

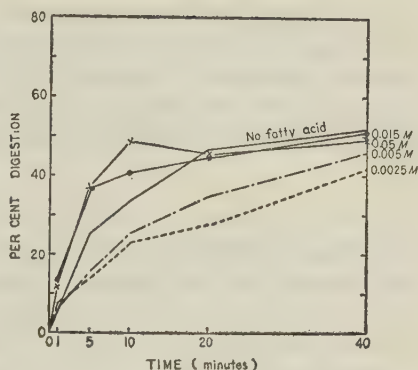


FIG. 4. Effect of C₁₂ fatty acid added on the digestion of serum albumin by trypsin.

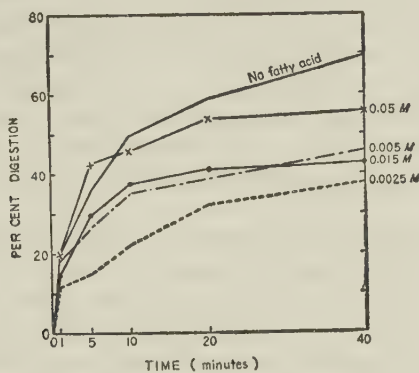


FIG. 5. Effect of C₁₄ fatty acid added on the digestion of serum albumin by trypsin.

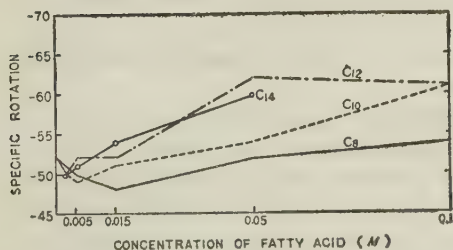


FIG. 6. The relationship between the specific rotation of serum albumin and the concentration of fatty acids.

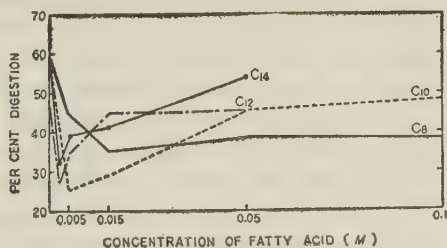


FIG. 7. The relationship between the per cent digestion of serum albumin and the concentration of fatty acids. Digestion time: 20 minutes.

The tryptic digestibility of serum albumin which was heat-treated in the presence of fatty acids was almost equal to that of heat-treated albumin in the absence of fatty acids.

3. *Change in the Optical Rotation of Serum Albumin by the Addition of Various Fatty Acids*—As shown in Figs. 6 and 7, the specific rotation of serum albumin solution decreased by the addition of fatty acid at lower concentrations but increased gradually with the increase of the concentration of fatty acids. These phenomena will be discussed later.

DISCUSSION

Horse serum albumin is, even in its native state, remarkably digestible with trypsin and its digestibility decreases upon the addition of fatty acids having carbon chain lengths within 8 to 14 (Figs. 2-5). No influence of fatty acids on the activity of trypsin is found at the concentration of 0.015 *M* of C_{12} -fatty acid. These results are consistent with that of Rice *et al.* (4), who stated that the heat denatured human serum albumin became less digestible by papain with the addition of fatty acids at lower concentrations.

As shown in Fig. 6, the laevorotation of serum albumin decreases by the addition of fatty acids tested at lower concentrations (0.0025, 0.005, 0.015 *M*) except C_{14} acid. Furthermore, as shown in Fig. 7, the digestibility by trypsin decreases to a definite level on addition of fatty acids in lower concentrations whereas it increases upon further addition of fatty acids. The concentration of each fatty acid at which the minimum laevorotation value of serum albumin is reached is equal to the concentration which gives the minimum tryptic digestibility.

It has been pointed out that the increase in laevorotation (5, 6) and the susceptibility to proteinases (7, 8) usually accompany the denaturation of proteins. According to Jirgensons' (9) report, the optical rotation and viscosity of crystallized bovine serum albumin decreased, when alcohols and salts were added to the albumin in relatively low concentrations. This decrease seemed to be associated with the changes in the molecular volume of the albumin and there was no denaturation. Changes in the optical rotation of serum albumin by additions of anionic azo dyes were investigated by Markus and Karush (11). Boyer *et al.* (2, 3) showed that human serum albumin combined with fatty acids to form a complex compound which had an increased thermal stability.

The author's results suggest the possible formation of complexes with fatty acids which have lower laevorotation values and are less susceptible to trypsin, and thus have a more compact structure than the native protein. These data are similar to those obtained with the serum albumins of other animals. Ovalbumin as reported in the previous paper (1) is remarkably different from serum albumin in those properties and, accordingly, they are probably different in their structure.

SUMMARY

1. Native horse serum albumin is remarkably digestible with trypsin compared with ovalbumin.

2. Horse serum albumin becomes less digestible with trypsin by the addition of C₈-C₁₄ fatty acids and the digestibility of serum albumin is not influenced remarkably by heating with fatty acids.

3. The laevorotation of horse serum albumin solution decreases by the addition of fatty acid at lower concentrations but increases at higher concentrations.

The author wishes to express her sincere thanks to Prof. S. Akabori and Prof. B. Maruo and Dr. H. Takahashi for their kind guidance and encouragement throughout this work.

REFERENCES

- (1) Kondo, M., *J. Biochem.*, **45**, 151 (1958)
- (2) Boyer, P. D., Lum, F. G., Ballou, G. A., Luck, J. M., and Rice, R. G., *J. Biol. Chem.*, **162**, 181 (1946)
- (3) Boyer, P. D., Ballou, G. A., and Luck, J. M., *J. Biol. Chem.*, **167**, 407 (1947)
- (4) Akabori, S., Amino acids and Proteins, Kyoritsu Press Co., Tokyo, P. 568 (1947)
- (5) Rice, R. G., Ballou, G. A., Boyer, P. D., Luck, J. M., and Lum, F. G., *J. Biol. Chem.*, **118**, 609 (1945)
- (6) Funatsu, K., *J. Agr. Chem. Soc. Japan*, **31**, 52 (1957)
- (7) Jirgensons, B., *Arch. Biochem. Biophys.*, **48**, 154 (1954)
- (8) Linderstrøm-Lang, K., Hotchkiss, R. D., and Johansen, G., *Nature*, **142**, 996 (1938)
- (9) Tazawa, Y., *Acta Phytochim.*, **14**, 79 (1944)
- (10) Jirgensons, B., *Arch. Biochem. Biophys.*, **57**, 376 (1955)
- (11) Markus, G., and Karush, F., *J. Am. Chem. Soc.*, **80**, 89 (1958)

PREPARATION OF P^{32} -LABELED ADENOSINE TRIPHOSPHATE BY HUMAN ERYTHROCYTES

By MASAMITI TATIBANA, MAKOTO NAKAO, KANJI MIYAMOTO,
TOYOZO SEKIGUCHI AND HARUHISA YOSHIKAWA

(From the Department of Physiological Chemistry and Nutrition,
Faculty of Medicine, University of Tokyo, Tokyo)

(Received for publication, October 1, 1958)

Human erythrocytes are exchanging their intracellular phosphate with inorganic orthophosphate in the plasma at a considerably rapid rate. An experiment carried out in our laboratory (1) has shown that when the erythrocytes were incubated for 30 minutes with P^{32} -orthophosphate added in the plasma, the acid soluble organic phosphate took up more than 10 per cent of P^{32} , of which about 40 per cent was incorporated in adenosinetriphosphate (ATP) and, besides, that the equilibration of specific activity between the two labile phosphate groups of ATP was reached in less than one minute, while very little incorporation into α -phosphate* was observed even after 30 minute incubation.

In addition to the above findings, the almost exclusive existence of adenine nucleotides in human erythrocytes (2, 3) and requirement of blood for neither substrate nor buffer provide a simple method for preparing P^{32} -labeled ATP.

The present communication deals with researches on optimal condition for the preparation.

EXPERIMENTALS

Preparation—1. A hundred milliliters of fresh heparinized blood was divided into six portions of 15 ml., each of which was taken in a 50 ml. centrifuge tube. To four of the portions were added 3 ml. of ACD (Acid Citrate Dextrose) solution, which is commonly employed as the diluent of blood in blood bank. Two of the four ACD blood samples were centrifuged at $1,300 \times g$ for 15 minutes and 8 ml. of the plasma was taken and discarded. Red cells were resuspended in the remaining plasma. In Table I were shown the conditions for incubation of the divided portions of the blood and the designations used hereafter to indicate them. In the other series of experiment, two portions of heparinized blood were incubated with P^{32} for 15 and 30 minutes.

2. Preincubation was carried out for all the portions at 37.5° for 30

* The three P atoms of ATP are expressed as α , β and γ , thus: adenosine- P^α - P^β - P^γ .

minutes. To each was added 0.5 ml. of P^{32} -orthophosphate solution containing about 45 μ c. of activity. The added radioactive phosphate had been previously hydrolyzed in 0.1 N HCl at 100° and neutralized with 1 N NaOH with phenolphthalein as internal indicator. This treatment had been shown to be necessary to destroy some contaminating phosphorus compounds other than orthophosphoric acid (4). No attention was paid to the isotonicity of the P^{32} solution because of its relatively small volume.

3. Periods of incubation were 30 and 60 minutes for each one of the three pairs of the blood. At the end of the time, 30 ml. of ice-cold physiological saline solution was added to the blood, followed by centrifugation at $1,300 \times g$. for 15 minutes. Plasma was discarded. Forty milliliters of cold saline was added to the packed cells and the washing was repeated.

4. To the packed cells, about 7 ml., was added 35 ml. of ice-cold 0.6 N perchloric acid. The mixture was stirred with a glass rod and after 10 minutes centrifuged at $4,000 \times g$. for 30 minutes in the cold.

TABLE I
Conditions for Incubation of the Divided Portions
of the Blood and Their Designations

	Incubation period with P^{32} -orthophosphate	
	30 minutes	60 minutes
Heparinized blood 15 ml.	H ₃₀	H ₆₀
Heparinized blood 15 ml. + ACD 3 ml.	C _{I 30}	C _{I 60}
Heparinized blood 15 ml. + ACD 3 ml. followed by centrifugation and removal of 8 ml. of the plasma	C _{II 30}	C _{II 60}

5. The extract obtained was neutralized with 5 N KOH using phenol red as internal indicator and the precipitate was removed by centrifugation in the cold. To the supernatant was added 1 ml. of 0.6 N perchloric acid to make the solution weakly acid. The solution was passed through a charcoal-Celite column 4×0.8 cm. (charcoal 150 mg. + Celite 150 mg.) by compressed air pressure. Preparation of charcoal-Celite column and its pretreatment with *sec.*-octanol was carried out according to the method of Threlfall (5). A slight modification was the use of usual Norit SX 30 and Hyflo Super Cell instead of Norit SX 30 Special and Celite 535.

6. After passing 1 ml. of water through the column, the adsorbed nucleotides were eluted out with 10 ml. of 0.05 N NH_4OH in 50 per cent ethanol-water, to which a trace of *sec.*-octanol had been added in some cases.

7. The eluate was diluted to 150 ml. with water and passed through

Dowex-1-Cl (X-2) column 5.0×0.7 cm. at a flow rate of about 1 ml. per minute. After washing with 50 ml. of water, elution was carried out by the following solutions (6); (a) $1/100$ N HCl + $1/50$ M NaCl 250 ml., (b) $1/100$ N HCl + $1/25$ M NaCl 100 ml. and (c) $1/100$ N + $1/15$ NaCl 200 ml.. The eluate by (b) and (c) was collected as fractions of 10 or 20 ml.

8. On each fraction ultraviolet absorbancy at $260\text{ m}\mu$ and radioactivity were measured. The greater part of ATP was eluted in the fractions (c).

9. The fractions of ATP were collected and neutralized with concentrated ammonia water and diluted with two volumes of water. The resulting solution was passed through Dowex-1-Cl (X-2) column 1.5×0.7 cm. Then followed the elution of ATP with 5 ml. of $1\text{ }N$ HCl in the cold. The eluate was immediately neutralized with $5\text{ }M$ KOH using phenolphthalein as internal indicator. Addition of three drops of 25 per cent barium acetate and one volume of 95 per cent ethanol followed. The mixture was left in a refrigerator for more than one hour. The precipitate of the barium salt was collected by centrifugation.

10. A portion of the barium salt was treated with Amberlite cation exchanger IR-120 (H^+) and was applied on Toyo Roshi No 53 paper for ascending paper chromatography. As the developing solvent n -butyric acid: $0.5\text{ }N$ NH_4OH , 20:12 (7) was used. Radioautography of the paper chromatogram was carried out using Fuji No Screen 200 X-ray film. Phosphorus was determined by a modified method of Horecker (8). The determination of ribose was made by the method of Mejsbaum (9).

Radiochemical Purity of P^{32} -ATP Prepared—Fig. 1 shows an example of the

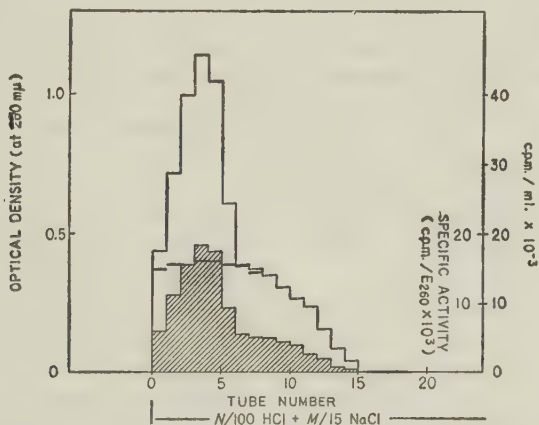


FIG. 1. Ion exchange chromatogram of ATP fraction ($C_{11\ 30}$).

Open columns represents optical density at $260\text{ m}\mu$ and hatched column represents radioactivity. AMP, ADP and most of the other nucleotides were eluted out before the ATP fractions as described in the text.

anion exchange chromatograms. A constant value of the ratios of radioactivity to E_{260} of the fractions was one of the indications for radiochemical

purity of P^{32} -ATP eluted there.

On the radioautogram (Fig. 2) appeared a dense spot of ATP accompanied by very faint spots of ADP and inorganic orthophosphate. The presence of the latter two spots might be due to the partial decomposition of ATP during the procedures for paper chromatography.

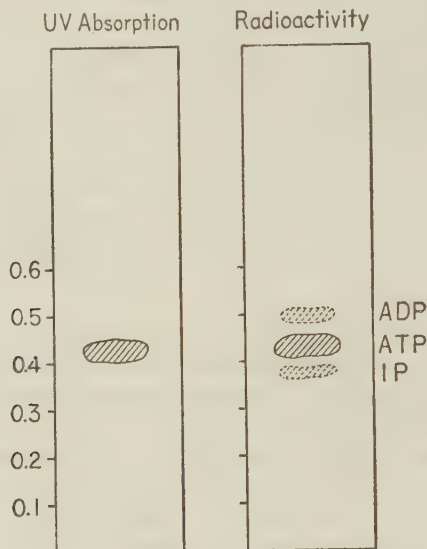


FIG. 2. Paper chromatogram and its radioautogram of ATP^{32} prepared.

Ascending paper chromatography was carried out with the developing solvent *n*-butyric acid:0.5 *N* NH_4OH , 20:12.

Chemical Purity of ATP Prepared—The ultraviolet absorption spectra at both acid and alkaline pH were identical with those of authentic ATP. The molar ratio of adenine:ribose:phosphorus was 1:1:3. On the paper chromatogram was observed only one spot of ATP by the ultraviolet detection method of Carter (9). Upon partial hydrolysis, two additional spots of ADP and AMP appeared, but no other nucleotide was detected.

Radioactivity of α -Phosphate—In Fig. 3 is shown one of the hydrolysis curves of P^{32} -ATP obtained. It reaches a plateau in less than 20 minutes and the portion between 20 and 30 minutes goes up with a very small value of gradient. The extrapolation of linear portion to $t=0$ will give the percent value of the radioactivity taken up by α -phosphate to the total sum of the radioactivity of all the three phosphates. This method was based upon the difference found in behaviors of the three phosphate groups of ATP toward acid hydrolysis (10). However, the values obtained in this way will be the possible maximum for the radioactivity of α -phosphate, because a small fraction of labile phosphates might remain unhydrolyzed after twenty minute hydrolysis.

Recovery of P³² in ATP and Pattern of Labeling—From the measurement of the fractions (c), the recovery rates of P³² in ATP were calculated. The results were shown in Table II along with the pattern of labeling of the three phosphorus atoms. The latter was calculated from the radioactivity of α -phosphorus atom, on the basis of the fact that both β - and γ -phosphate groups possess a same specific activity at the incubation periods of such length (I).

The experiment C_{II 60} gave the highest P³² recovery, about 25 per cent. The ratio of specific activity of α -phosphorus to β - or γ -phosphorus was less than 1:30.

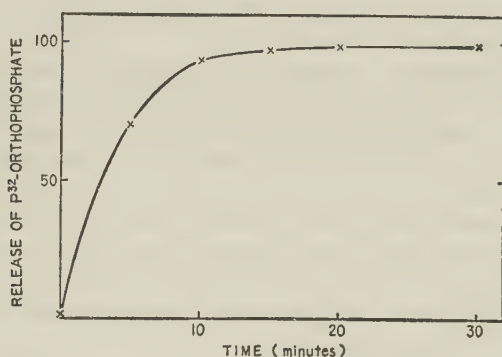


FIG. 3. Acid hydrolysis of ATP³² obtained.

The sample analyzed here was the fraction No. 6 of the experiment C_{II 30}. One milliliter of the sample was taken in each of six stoppered test tubes. To each, 1 ml. of 2 *N* H₂SO₄ was added and the mixture was kept in a boiling water bath for the time period indicated. Then followed addition of 1 ml. of 2 per cent sodium molybdate and 2 ml. of isobutanol and vigorous shaking. Both organic solvent and aqueous layer was assayed for P³².

TABLE II
Specific Activities of ATP³² Prepared and Recoveries of P³²

Expt.	Conditions for incubation	P ³² recovery (per cent)	Specific activities of the three phosphorus and their ratios (c.p.m./ μ g. P)
No. 1	H ₃₀	1.7	7:1/2* 1460:1/2 1460 1:104:104
	H ₆₀	9.3	107:1/2 5200:1/2 5200 1:24:24
	C _{I 30}	3.8	40:1/2 1620:1/2 1620 1:20:20
	C _{I 60}	3.8	60:1/2 2340:1/2 2340 1:20:20
	C _{II 30}	11.4	200:1/2 8100:1/2 8100 1:20:20
	C _{II 60}	26.3	270:1/2 14000:1/2 14000 1:26:26
No. 2	H ₁₅	2.1	50:1/2 5600:1/2 5600 1:120:120
	H ₃₀	3.5	170:1/2 8600:1/2 8600 1:50:50

* The number 1/2 means that the specific activities of β - and γ -phosphorus were not measured individually.

DISCUSSION

The present method for preparing P^{32} -labeled ATP has several advantages. Simplicity is the first one. Requirement of blood for neither buffer nor substrate and nearly exclusive existence of adenine nucleotides in human erythrocytes simplified the procedures for the present method. The second is its easiness. Stability of the red cells assures a constant yield. The third is the relatively high recovery rates of P^{32} in ATP, in addition to the advantages described above. Preparation of P^{32} -labeled ATP with rat liver mitochondria was reported to give a maximum recovery of 90 per cent (11). However, such a high yield appears not to be a constant one because of the less stability of mitochondrial preparation.

Further simplification of the present method is possible by omitting charcoal treatment. But this will necessitate more cautious operation of subsequent ion exchange chromatography for the complete separation of ATP from 2,3-diphosphoglyceric acid, which, in the method outlined here, was completely removed by the charcoal operation. The use of ion exchange chromatography under the formate system according to Hurlbert and Potter (12) makes possible the separation of ATP from the other acid soluble phosphorus compounds of human erythrocytes in one procedure (2, 3). However, in this case, the recovery of the nucleotide from the effluent needs rather tedious procedures.

Blood from some other animal, such as rabbit, may be employed as well as human blood. However, preliminary experiments will be necessary, because remarkable differences have been found among the erythrocytes from different species in their permeability to inorganic orthophosphate (13, 14) and content of ATP (15).

If a slightly higher ratio of the specific activity of α -phosphorus to β - and γ -phosphorus is permissible, the P^{32} recovery might nearly be doubled by employing a longer incubation. Preserved blood two or three days old can be used as well as fresh blood.

SUMMARY

ATP ^{32} labeled at β and γ position is easily prepared by incubating human erythrocytes with P^{32} -orthophosphate. Optimal conditions for the method was studied. Need for neither buffer nor substrates and stability of the blood as a biological system are the main advantages of the present method. The recovery of P^{32} in ATP reached about 25 per cent and the ratio of the specific activity of α -phosphorus to that of β - or γ -phosphorus was less than 1:20.

REFERENCES

- (1) Tatibana, M., *J. Japan. Biochem. Soc. (in Japanese)*, **30**, 667 (1958)
- (2) Banderheiden, B. S., Huennekens, F. M., and Gabrio, B. W., *Fed. Proc.*, **17**, 327 (1958)

- (3) Bartlett, G. R., Savage, E., Hughs, L., and Marlow, A. A., *J. Appl. Physiol.*, **6**, 51 (1953)
- (4) Tatibana, M., and Yoshikawa, H., *Radioisotopes (Japan)*, **7**, 57 (1958)
- (5) Threlfall, C. J., *Biochem. J.*, **65**, 694 (1957)
- (6) Cohn, W. E., and Carter, C. E., *J. Am. Chem. Soc.*, **72**, 4273 (1950)
- (7) Omura, T., and Fukushi, S., *J. Agr. Chem. Soc. Japan (in Japanese)*, **27**, 516 (1953)
- (8) Horecker, B. L., Ma, T. S., and Haas, E., *J. Biol. Chem.*, **136**, 775 (1940)
- (9) Mejbaum, W. Z., *Z. physiol. Chem.*, **238**, 117 (1939)
- (10) Le Page, G. A., *Manometric Techniques*, ed. by Umbreit, Burris, and Stauffer, University of Wisconsin Press, Minneapolis, p. 271, (1957)
- (11) Kielly, W., in *Methods in Enzymology*, ed. by Colowick and Kaplan, Academic Press, New York. Vol. **4**, P. 853, (1957)
- (12) Hurlbert, R. S., Schmitz, H., Brumm, A. F., and Potter, V. R., *J. Biol. Chem.*, **209**, 23, (1954)
- (13) Levi, H., *Kgl. Danske Videnskab. Selskab Mat. fys. Medd.*, **23**, No. 10 (1954)
- (14) Schambye, P., *Nutrition Abstr. and Revs.*, **23**, 110 (1953)
- (15) Rapoport, S., and Guest, M., *J. Biol. Chem.*, **138**, 269 (1941)

THE STRUCTURE OF CYTOCHROME C

V. DIAZO-COUPPLING AND IODINATION OF CYTOCHROME C*

By HISAYUKI ISHIKURA, KENJI TAKAHASHI, KOITI TITANI

(From the Department of Chemistry, Faculty of Science,
University of Tokyo, Tokyo)

AND SHIGEKI MINAKAMI

(From the Department of Physiological Chemistry and Nutrition, Faculty of
Medicine, University of Tokyo, Tokyo)

(Received for publication, October 3, 1958)

In the previous papers (1, 2) the authors have reported the modification of amino-groups of cytochrome c effected by two methods, acetylation and guanidination. The acetylated derivative showed several properties different from those of the native one, for example, it was autoxidizable and did not accelerate oxygen uptake in the succinic oxidase system. On the other hand, the guanidinated derivative showed the same biological activities and characteristic properties as the native one except the decrease of stability. From these results, it was concluded that one of the roles of the amino-groups in cytochrome c lies in their cationic character.

In the present paper, the imidazole groups of cytochrome c were modified by diazo-coupling and by iodination. The changes of its catalytic activities as well as the characteristic properties caused by these modifications were examined. *p*-Diazobenzenesulfonic acid was used as a diazo-coupling reagent. Iodination was carried out by using radioactive iodine and the number of iodine atoms incorporated was estimated by determining the radioactivity.

EXPERIMENTAL AND RESULTS

Cytochrome c—Cytochrome c was extracted from the horse heart muscle and purified by the chromatographic method as described in the previous paper (1).

Experiment 1. Cytochrome c Modified by Diazo-coupling

Treatment with p-Diazobenzenesulfonic Acid—Cytochrome c was dissolved in 0.33 *M* phosphate buffer of pH 7.6. Appropriate amounts of *p*-diazobenzenesulfonic acid (2–300 moles of the reagent per mole of the protein) were added to the solution of cytochrome c. After standing at room temperature

* A part of this study was presented at the "International Symposium on Enzyme Chemistry" October 1957, Tokyo.

for half an hour, the reaction mixture was dialysed against the same buffer for 24 hours. The dialysed sample was divided into two portions; the first portion was diluted for spectrophotometric measurement and the other for the assay of protein concentration. The spectrum of oxidized form was examined in the wavelength range of 450–600 $m\mu$. After reduction with sodium dithionite, the spectrum of the reduced form was examined in the range of 500–600 $m\mu$. The optical densities were corrected on the basis of the protein concentrations determined by the micro-Kjeldahl method.

As shown in Fig. 1, the absorption band at 550 $m\mu$ shown by the reduced form of cytochrome c decreased as the reaction proceeded. The coupling is shown by an increase of absorption in the range of 450–500 $m\mu$ in the oxidized state. The capacity of reversible oxido-reduction, *i.e.*, the electron transferring activity, was found to be completely abolished by diazo-coupling.

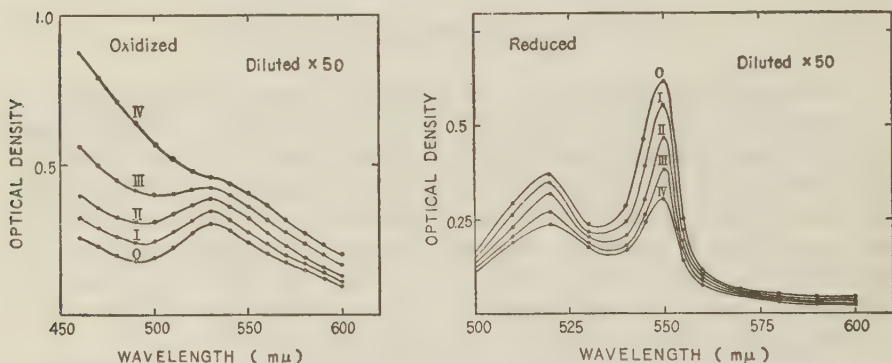


FIG. 1. Changes in absorption spectra of cytochrome c caused by diazo-coupling with *p*-diazobenzenesulfonic acid.

0: 0.1 μM cytochrome c.

I: 0.1 μM cytochrome c + 0.27 μM diazobenzenesulfonic acid.

II: 0.1 μM cytochrome c + 0.54 μM diazobenzenesulfonic acid.

III: 0.1 μM cytochrome c + 1.35 μM diazobenzenesulfonic acid.

IV: 0.1 μM cytochrome c + 2.7 μM diazobenzenesulfonic acid.

Reaction in 0.33 M phosphate buffer, pH 7.6 at room temperature. Reaction time, 30 minutes. Reaction mixture was dialysed against the same buffer.

Experiment 2. Cytochrome c Modified by Iodination

Treatment with Iodine—Cytochrome c (68 mg., 7.0 μM) was dissolved in 20 ml. of 0.6 M phosphate buffer of pH 7.0. Ten milliliters of 1/100 M solution of iodine in approximately 0.02 M potassium iodide containing carrier-free radioactive iodide (NaI^{131}) were added and the whole mixture was kept at 0°. At suitable intervals 3 ml. each of aliquots were pipetted out and 1 ml. of 0.1 N sodium thiosulfate solution was added to stop the reaction. Each aliquot was divided into two portions, the first portion (0.5 ml.) being diluted and used for the spectrophotometric assay, and the second portion (3.5 ml.) being dialysed against 0.5 per cent sodium chloride

solution with stirring for 48 hours, changing the saline for several times. After the measurement of radioactivity, it was used for the study of catalytic and other properties and for the determination of nitrogen content by the micro-Kjeldahl method. The experiment using acetate buffer of pH 5.0 was also carried out. In this case, however, the protein was precipitated and only a small amount of iodine was found to be incorporated into the protein.

Determination of the Number of Iodine Atoms Incorporated into the Protein—The number of iodine atoms incorporated into the cytochrome c molecule was calculated from the specific activity of the iodine atom in the solution, the radioactivity of the dialysed iodinated cytochrome c and the concentration of the protein. The rapid equilibration of iodine molecule with iodide ion has been known. The radioactivity of the iodine solution used was estimated using a well-type scintillation counter (Scientific Research Institute, Tokyo). Analysis of iodide concentration in the solution was carried out according to the method of Dietz and Margosches (3).

Chromatography of Iodinated Amino Acids—The iodinated protein was hydrolyzed with 2 *N* sodium hydroxide or by applying pancreatic protease. The hydrolysate, acidified to pH 1.0 with hydrochloric acid, was extracted three times with *n*-butanol saturated with 0.1 *N* hydrochloric acid. The *n*-butanol solutions were evaporated to dryness *in vacuo* and spotted on paper (Tokyo Filter paper No. 53) to obtain a two dimensional ascending paper chromatogram using *n*-butanol-2 *N* NH_4OH and *n*-butanol-5 per cent acetic acid as developing solvents (4). Radioautogram using an X-ray film, showed the spots corresponding to iodide, di-iodotyrosine and thyroxine.

Spectrophotometric Study—The diluted solution of the iodinated cytochrome c was reduced by the addition of sodium dithionite. The intensity of the absorption at 550 $\text{m}\mu$ was measured and expressed as percentage of the original absorption. For the determination of the affinity towards carbon monoxide, pure carbon monoxide was passed for 5 minutes through the solution containing cytochrome c in the reduced state. The carbon monoxide binding affinity was calculated according to Tsou (5). A small portion of the solution was reduced with hydrogen palladium black to observe the autoxidizability of the pigment. With the iodinated sample, the α -band of cytochrome c soon disappeared on aeration.

Measurement of Catalytic Activity—The electron transferring ability of iodinated cytochrome c in a succinic oxidase system was estimated manometrically using the system of rat liver homogenate as described in a previous paper (1). The values were corrected on the basis of protein concentration and expressed as percentage of the original activities.

Estimation of Hydroxylamine Reductase Activity—This activity was estimated by measuring the time necessary for the decolorization of reduced methylviologen using Thunberg tubes. Reaction mixture contained 0.023 *M* phosphate buffer of pH 7.0, 2×10^{-3} *M* reduced methylviologen, 0.03 *M* hydroxylamine hydrochloride, and 3×10^{-8} *M* of cytochrome c.

The results of iodination are summarized in Table I. As the incorporation of iodine into cytochrome *c* molecule proceeded, the affinity towards carbon monoxide rapidly appeared, and at the same time the electron transferring activity was lost. The appearance of autoxidizability paralleled with them, whereas little change was observed in the intensity of the

TABLE I
Iodination of Cytochrome c

Sample No.	Reaction time (min.)	Number of iodine atoms incorporated per molecule of cytochrome <i>c</i>	Intensity of absorption at 550 m μ (%)	Autoxidizability	CO-binding affinity (%)	Catalytic activity (%)
0	0	0	100	—	15.3	100
1	0.5	2.71	99	+	72.8	30.0
2	1	3.94	104	+	82.5	11.0
3	2	4.44	101	+	91.0	0
4	5	5.39	100	+	90.0	0
5	15	5.80	96	+	96.0	0
6	30	6.47	93	+	97.0	0
7	60	6.56	94	+	90.0	0

absorption at 550 m μ in the reduced state. As for hydroxylamine reductase activity, the protein which had completely lost its catalytic activity in a succinic oxidase system by iodination for 15 minutes decolorized reduced methyl-viologen in a few seconds, whereas 150 seconds were necessary with the native one.

DISCUSSION

p-Diazobenzenesulfonic acid has been known to react with imidazole groups and phenolic groups of proteins. It is stated by Fraenkel-Conrat that imidazole groups of most proteins react with diazo-coupling reagent at least as fast as do phenolic groups (6). On the other hand, there exist some 'obscurities with the specificity of iodination of proteins. According to Fraenkel-Conrat (6), in most proteins the iodination is comparatively specific to phenolic groups but in some proteins histidine residues react readily with iodine.

A difference was observed in experiment between the diazo-coupling and iodination of cytochrome *c*; the distinct decrease of the absorption at 550 m μ in the reduced state was brought about by the diazo-coupling, while the iodination caused only slight decrease of the absorption even when 6 atoms of iodine were incorporated into the protein molecule.

It is well established that the absorption band at 550 m μ of the reduced

cytochrome c is due to the histidine-hemochrome structure of cytochrome c, namely the structure of the imidazole groups coordinated to the heme iron (7). Therefore, the findings mentioned above may be interpreted in the following way. The imidazole groups as well as the phenolic groups may be modified by reaction with the diazo-coupling reagent. This modification caused a disruption of histidine-hemochrome structure and resulted in the decrease of the absorption at $550\text{ m}\mu$. On the other hand, the reaction of iodine may be confined to the phenolic groups of cytochrome c in the experimental conditions mentioned above. Accordingly the absorption at $550\text{ m}\mu$ was maintained at almost the same value as the original one. Furthermore, the incorporation of about 6 atoms of iodine after 60 minutes of iodination well coincides with the fact that the horse heart muscle cytochrome c contains 3 tyrosine residues (8, 9).

As mentioned above, the electron transferring activity was lost as iodination proceeded. This suggests that on account of the incorporation of iodine into the tyrosine residues, the hydrogen bonds formed by the phenolic groups might be broken. Thus, the stereochemical structure of cytochrome c would be disrupted and simultaneously the ability to act as an electron carrier would be lost. This supposition seems to be supported by the fact that the appearance of the carbon monoxide binding affinity and that of the autoxidizability paralleled with the loss of the biological activity.

SUMMARY

1. Cytochrome c was modified by applying to it a diazo-coupling reagent, *p*-diazobenzenesulfonic acid. The decrease of the absorption at $550\text{ m}\mu$ in the reduced state of cytochrome c was observed, as the reaction proceeded. The electron transferring activity of cytochrome c was found to be abolished.

2. Cytochrome c was iodinated and the number of iodine atoms incorporated into the protein was quantitatively estimated with the aid of radioactive iodine. The carbon monoxide binding affinity appeared as iodination proceeded. The autoxidizability also appeared, simultaneously with the disappearance of the electron transferring activity in a succinic oxidase system. The absorption at $550\text{ m}\mu$, however, was maintained at almost the same level as the original one even when 6 atoms of iodine were incorporated into the protein molecule.

3. The difference between the results of the two types of modifications was discussed. It was inferred that with diazo-coupling the imidazole as well as the phenolic groups might have been modified, while the reaction of iodine be confined to the phenolic groups, in the experimental condition described in this paper.

The loss of electron transferring activity caused by iodination was considered to be a result of disruption of the stereochemical structure of cytochrome c.

The authors express their cordial thanks to Prof. S. Akabori and Prof. H. Yoshikawa for their constant interests and encouragements during this work.

REFERENCES

- (1) Minakami, S., Titani, K., and Ishikura, H., *J. Biochem.*, **45**, 341 (1958)
- (2) Takahashi, K., Titani, K., Furuno, K., Ishikura, H., and Minakami, S., *J. Biochem.*, **45**, 375 (1958)
- (3) Quoted from Treadwell, F. P., and Hall, W. T., *Analytical Chemistry*, John Wiley and Sons, New York, Vol. **II**, p. 603 (1942)
- (4) Roche, J., Lissitsky, S., and Michel, R., *Methods of Biochemical Analysis*, Interscience Publishers, New York, Vol. **I**, p. 243 (1954)
- (5) Tsou, C. L., *Biochem. J.*, **49**, 362, 367 (1951)
- (6) Fraenkel-Conrat, H., *Methods in Enzymology*, Academic Press, New York, Vol. **IV**, p. 247 (1957)
- (7) Theorell, H., and Akeson, A., *J. Am. Chem. Soc.*, **63**, 1804 (1941); Ehrenberg, A., and Theorell, H., *Acta Chem. Scand.*, **9**, 1193 (1955)
- (8) Nunnikhoven, R., *Biochim. et Biophys. Acta*, **28**, 108 (1958)
- (9) Takahashi, K., Titani, K., and Minakami, S., *J. Biochem.*, in press

PURIFICATION AND PROPERTIES OF A BACTERIAL DEOXYRIBOSE TRANSFERASE*

By MASAYUKI KANDA AND YASUYUKI TAKAGI

(From the Biochemical Department, Medical School,
Osaka University, Osaka)

(Received for publication, October 10, 1958)

Several deoxyribosides can serve equally well as growth-promoting substances for certain bacterial species, suggesting that deoxyriboside provided in the growth medium is easily converted into the other deoxyribosides during deoxyribonucleic acid synthesis by the cells (1-4). The rapid inter-conversion of deoxyribosides in such organisms was first demonstrated by Mc Nutt (5), who observed the transfer of the deoxyribosyl group from one purine or pyrimidine to another in the presence of enzyme preparations from several species of *Lactobacilli*. The enzymes concerned do not require phosphate or arsenate, and some of the properties of the *L. helveticus* enzyme were recently studied (6). Similar enzymes have also been reported from extracts of *Escherichia coli* B (7).

This paper reports on the purification and properties of deoxyribose transferase from *Lactobacillus delbrueckii*. It has been shown that this enzyme provides a useful tool for the liberation and determination of the deoxyribose moiety linked to pyrimidine in deoxyribonucleic acid.

METHODS

Materials—Adenine, guanine, hypoxanthine, xanthine, uric acid, cytosine, thymine, deoxyadenosine, deoxycytidine and thymidine were obtained from Nutritional Biochemicals Corp. 6-Mercaptopurine, 2,6-diaminopurine and 8-azaguanine were generously furnished by Dr. K. Tanaka of the Research Institutes of Takeda Pharmaceutical Co., Ltd. Kinetin was a gift of Dr. S. Okumura of Tokushima University.

The organism used in these experiments was *Lactobacillus delbrueckii* 9649 (American Type Culture Collection), provided by the Institute for Fermentation, Osaka. The cells were grown as previously described (8), harvested at 2° with a Sharples supercentrifuge, and the packed cells were stored at the frozen state until needed.

Analytical Methods—Transfer of deoxyribose was followed in two ways. With either thymidine as donor or thymine as acceptor of the carbohydrate moiety, the spectrophotometric procedure was used; thymine formed or disappeared was measured by its absorption at 300 mμ at alkaline pH (9, 10). In cases where transglycosidation of deoxyribosyl group from purine nucleosides to pyrimidine bases, or from pyrimidine nucleosides to purine bases was measured, the analytical procedure included a differentiation between purine

* Presented at the 30th Annual Meeting of the Japanese Biochemical Society (Kyoto, July, 1957).

and pyrimidine deoxyribosides based on the relative resistance of the latter toward acid (11). For example, since the purine deoxyriboside is readily hydrolyzed by acid to purine and deoxyribose which gives the diphenylamine reaction, while the pyrimidine derivative is stable, starting with a pyrimidine deoxyriboside and a purine base, the formation of the purine deoxyriboside was followed by the appearance of diphenylamine-reactive deoxyribose (12). Protein was determined spectrophotometrically by the method of B  cher (13).

Enzyme Assay—In the routine assay of deoxyribose transferase, thymidine was used as the sugar donor, and adenine as the acceptor. The reaction mixture contained 0.2 μ mole of thymidine, 0.2 μ mole of adenine, 0.04 ml. of 0.5 *M* phosphate buffer at pH 6.1 and suitable dilutions of enzyme in a total volume of 0.2 ml. After incubation for 15 minutes at 37°, the entire reaction mixture was made alkaline by the addition of 3.0 ml. of 0.3 *N* NaOH and the increase in optical density at 300 *m* μ was measured in a Beckman spectrophotometer.

A unit of enzyme activity was defined as the amount of enzyme required to cause a change in optical density of 1.0 per 15 minutes. Specific activity was the number of units per mg. of protein. Under the above conditions, the reaction rate was found to be linear for at least 30 minutes and directly proportional to the enzyme concentration (Fig. 1).

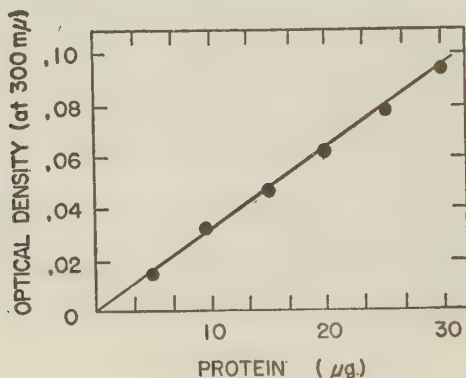


FIG. 1. Proportionality of reaction rate to quantity of deoxyribose transferase. The assay conditions are described in the text. The enzyme solution was Ammonium Sulfate Fraction of specific activity of 3.1 units per mg. The rate of transfer was indicated by the change of optical density at 300 *m* μ for thymine.

RESULTS

Purification Procedure

Extraction—The first steps in the procedure were adapted from the method of McNutt (5). 7 g. of frozen cells were treated with alumina as previously described (8), and the resulting paste was extracted with 60 ml. of 0.05 *M* citrate buffer, pH 6.0. The insoluble residue was removed by centrifugation at 8,000 $\times g$ for 10 minutes and the supernatant solution was dialyzed against running distilled water at 5° overnight (Crude Extract, Table I).

Manganese Fraction—68 ml. of Crude Extract was treated with 0.02 volumes of 1 *M* MnCl_3 , allowed to stand at 0° for 30 minutes with occasional stirring, and centrifuged at $8,000 \times g$ for 10 minutes. The heavy precipitate containing nucleoprotein was discarded (Mn^{++} Fraction, 66 ml.).

Ammonium Sulfate Fraction—54 ml. of cold saturated ammonium sulfate solution was added to 66 ml. of the Mn^{++} Fraction and the precipitate was discarded. A second fraction was collected by the addition of 12 g. of ammonium sulfate to the supernatant solution, and this precipitate was dissolved in distilled water to a total volume of 30 ml. (Ammonium Sulfate Fraction, 30 ml.).

TABLE I

Purification of Deoxyribose Transferase from L. delbrueckii

Fraction	Total units	Specific activity (units per mg.)
Extract	360	0.5
Mn^{++} Fraction	324	1.1
Ammonium Sulfate Fraction	240	3.1
Acetone Fraction	131	9.5

Acetone Fraction—To a 10 ml. portion of the Ammonium Sulfate Fraction was added 4.3 ml. of cold acetone (-10°). During the addition, which required 2 minutes, the mixture was cooled in a freezing bath, to a final temperature of -10° . The precipitate was removed by centrifugation at $1,600 \times g$ for 5 minutes at -10° . A second fraction was obtained in the same way by the addition of 5.7 ml. of acetone to the supernatant solution and the precipitate dissolved in 10 ml. of distilled water. The activity was usually present only in the second fraction (Acetone Fraction, 30 ml.).

Properties of the Enzyme

Effect of pH—With either thymidine or deoxycytidine as the donor of the carbohydrate moiety, and adenine as the acceptor the pH optimum was similar and was about pH 6.0 (Fig. 2). Under the assay conditions the reaction velocity decreased rapidly below this pH, but changed little between pH 6.0 and 7.0. The activity of the enzyme was not influenced by the buffer tested at the same pH, and the transfer occurred equally well in the absence of phosphate buffer (Fig. 3).

Stability—At the Ammonium Sulfate Fraction stage, the enzyme was moderately stable, and could be stored in the deep freeze for at least a month with little or no loss of activity, though it lost appreciable activity in 3 months. However, the final enzyme preparations were less stable and the activity was reduced to approximately one half in a month on storage at the frozen state. The addition of glutathione was found to be essential

to retain enzyme activity during storage, and preparations in the presence of 0.15 *M* glutathione showed negligible loss of activity after more than 3 months when kept frozen.

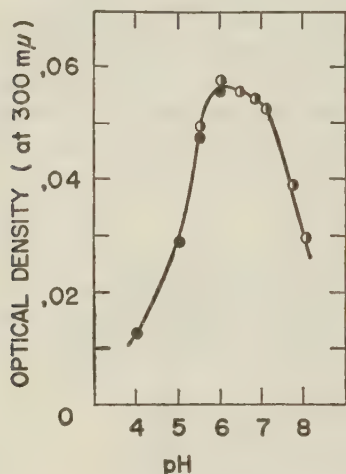


FIG. 2. Variation of deoxyribose transferase activity with pH. The reaction mixtures contained 0.11 ml. of 0.05 *M* buffer solution, 0.4 μ mole of thymidine, 0.4 μ mole of adenine and 0.01 ml. of enzyme (Ammonium Sulfate Fraction, specific activity=3.1 units per mg.) in a total volume of 0.20 ml. Incubation was for 15 minutes at 37°. Buffers used: pH 4.0 to 6.0, citrate, ●; pH 5.5 to 8.0, phosphate, ○.

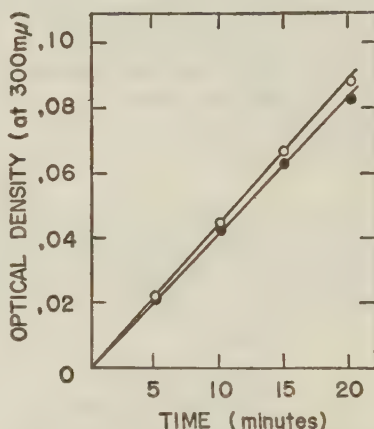


FIG. 3. Activity of deoxyribose transferase with thymidine in various buffers. Each reaction was carried out at 37° in a total volume of 1.5 ml. containing 3 μ moles of adenine, 3 μ moles of thymidine, 0.3 ml. of the buffer indicated (0.5 *M*) and 0.075 ml. of purified enzyme (Acetone Fraction, specific activity=9.5 units per mg.). 0.2 ml. aliquots were removed at the points shown and thymine assayed as described in the text. ○, phosphate, pH 6.0; ●, citrate, pH 6.0.

Equilibrium Measurements—The equilibrium of the reaction with equimolar quantities of thymidine as donor and adenine as acceptor is shown in Fig. 4. Under these conditions, equilibrium was established within 6 hours, and the final mixture contained about 80 per cent of the total deoxyribose as purine derivative. The same equilibrium mixture was obtained with deoxyadenosine as donor and thymine as acceptor. In similar experiments with deoxycytidine in place of thymidine, at equilibrium the purine deoxyriboside content was almost the same.

The content of purine deoxyriboside increased, if a considerable excess of acceptor was employed and the incubation period was 18 hours (Table II). For instance, in the presence of the five times as much adenine as pyrimidine deoxyriboside, more than 96 per cent of the deoxyribose was transferred from the pyrimidine nucleoside to adenine.

Substrate Specificity and Enzyme Affinity—Various purines were tested for their ability to react with deoxycytidine to give purine deoxyribosides.

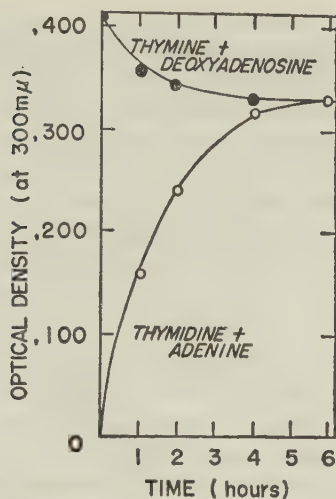


FIG. 4. Equilibrium measurements at 37° with thymidine and deoxyadenosine as substrates. The following conditions were used: 3 μ moles of thymidine or deoxyadenosine was incubated with 0.075 ml. of enzyme preparation (Acetone Fraction, specific activity =9.1 units per mg.) in the presence of 3 μ moles of adenine or thymine and 0.3 ml. of 0.5 *M* phosphate buffer (pH 6.1) in a total volume of 1.5ml. 0.2ml. aliquots were taken as shown and analyzed for thymine by spectrophotometric analysis.

TABLE II

Equilibrium Measurements with Thymidine and Deoxycytidine at Various Concentrations

The reaction mixtures containing 0.04 ml. of 0.5 *M* phosphate buffer, 1.0 μ mole of adenine, 0.02 ml. of enzyme (Acetone Fraction, specific activity=9.1 units per mg.) and thymidine or deoxycytidine at concentration indicated in a total volume of 0.2 ml. were incubated under toluene for 18 hours at 37°, and analyzed for deoxyadenosine by the diphenylamine method.

Adenine (μ mole)	Thymidine (μ mole)	Deoxycytidine (μ mole)	Per cent of transferred deoxyribose (%)
1.0	0.2		>96
1.0	0.3		96
1.0	0.5		94
1.0	0.7		87
1.0		0.3	>96
1.0		0.5	96
1.0		0.7	95

The comparative rate of formation of various purine deoxyribosides is shown in Table III, in which the rate of transfer to adenine is represented by 100. Adenine, xanthine and kinetin at a concentration of $2 \times 10^{-3} M$ gave nearly equal activity, while guanine, hypoxanthine, 6-mercaptopurine and 2,6-diaminopurine at the same concentration were about one half as effective as adenine. Uric acid, and 8-azaguanine were less than one-tenth as effective as adenine. These results indicate that the enzyme participates in the transfer of deoxyribose to a considerable number of bases, as pointed out by McNutt (5). However, ribosides did not enter into the exchange reaction.

TABLE III

Comparative Rate of Transglycosidation from Deoxycytidine to Natural and Synthetic Purine Bases

The reaction mixture (0.2 ml.) contained 0.4μ mole of deoxycytidine, 0.4μ mole of acceptor, 0.04 ml. of 0.5 M phosphate buffer at pH 6.1, 0.01 ml. of purified enzyme (Ammonium Sulfate Fraction, specific activity=3.1 units per ml.) After 15 minutes at 37° the mixture was analyzed for purine deoxyriboside by the Dische's method.

Acceptor	Comparative rate (%)
Adenine	100
Guanine	62
Hypoxanthine	36
Xanthine	71
Uric acid	9
6-Mercaptopurine	62
2,6-Diaminopurine	54
8-Azaguanine	6
Kinetin	75

DISCUSSION

McNutt observed that the interconversion of one deoxyriboside into another in several species of *Lactobacilli* could proceed in the absence of phosphate, indicating no participation of nucleoside phosphorylase in the exchange reaction (5). However a possibility was suggested that further purification of the enzyme might disclose phosphorylated intermediates. On the basis of the results described in the paper, this appears unlikely in the present case, since no phosphate requirement was detected either with crude or purified enzyme preparations. The reaction catalyzed by this enzyme, therefore was shown to be a phosphate-independent sugar transfer reaction, analogous to trans-*O*-glucosidase from *Pseudomonas saccharophila* (14), while there was no indication on a similar transfer of the ribosyl group.

More than 96 per cent of the sugar moiety was transferred from the pyrimidine deoxyriboside to adenine, if an excess of the purine base was provided. Thus it was shown that almost all of the deoxyribose linked to pyrimidine could be hydrolytically split by acid after it had been transferred to purine. Since the pyrimidine deoxyribose is relatively resistant to hydrolysis, the procedure is useful for the determination of pyrimidine deoxyribosides, and for separation of the deoxyribosyl moiety of these compounds, necessary during the isotopic studies.

Since a rather wide assortment of nitrogenous bases participate in the transfer reactions, this enzyme may be a relatively nonspecific deoxyribose transferase. Therefore, using this enzyme, it is possible to synthesize deoxyribose derivatives involving various purine analogues which might be expected to have more potential growth inhibition action than the free bases.

SUMMARY

1. Deoxyribose transferase has been purified about 20-fold from the extract of *Lactobacillus delbrueckii*. With the purified enzyme preparation no evidence for phosphate requirement has been found.

2. The enzyme catalyzes a reversible reaction: with initially equimolar quantities of pyrimidine deoxyriboside and adenine, at equilibrium about 80 per cent of the total deoxyribose is present as purine derivatives, and the content increases with the elevated levels of adenine added as acceptor of the deoxyribosyl group. A considerable number of purine bases participate in this transfer reaction.

3. The possibility that the deoxyribosyl group of pyrimidine deoxyribosides could be determined or isolated using this enzyme is discussed.

The authors wish to express their appreciation Dr. B. L. Horecker of the National Institutes of Health, Bethesda, Maryland and to Prof. K. Ichihara for his interest and encouragement in this work.

This work was supported by a grant for Fundamental Scientific Research of the Ministry of Education.

REFERENCES

- (1) Kitay, E., McNutt, W. S., and Snell, E. E., *J. Biol. Chem.*, **177**, 993 (1949)
- (2) Kitay, E., McNutt, W. S., and Snell, E. E., *J. Bacteriol.*, **59**, 727 (1949)
- (3) Hoff-Jørgensen, E., *J. Biol. Chem.*, **178**, 525 (1949)
- (4) Kocher, V., and Schindler, D., *Intern. Z. Vitaminforsch.*, **20**, 441 (1949)
- (5) McNutt, W. S., *Biochem. J.*, **50**, 384 (1953)
- (6) Roush, A. H., and Betz, R. F., *J. Biol. Chem.*, **233**, 261 (1958)
- (7) Lampen, J. O., in *Phosphorus Metabolism*, ed. by McElroy, W. D., and Glass, B., Johns Hopkins Press, Baltimore, Vol. 2, p. 363 (1952)
- (8) Takagi, Y., and Horecker, B. L., *J. Biol. Chem.*, **225**, 77 (1957)
- (9) Hotchkiss, R. D., *J. Biol. Chem.*, **175**, 315 (1948)
- (10) Carter, C. E., *J. Am. Chem. Soc.*, **73**, 1508 (1951)

- (11) Levene, P. A., and London, E. S., *J. Biol. Chem.*, **83**, 793 (1929)
- (12) Dische, Z., *J. Biol. Chem.*, **204**, 983 (1953)
- (13) Bücher, T., *Biochim. et Biophys. Acta*, **1**, 292 (1947)
- (14) Doudoroff, M., Barker, H. A., and Hassid, W. Z., *J. Biol. Chem.*, **168**, 725 (1947)

ON THE EFFECT OF BILE ACIDS ON CARBOHYDRATE METABOLISM

II. EXPERIMENTAL DIABETES INDUCED BY SODIUM CHOLATE

By ISAMU YANAGISAWA

(From the Department of Nutrition and Physiological Chemistry,
Faculty of Medicine, University of Tokyo, Tokyo)

(Received for publication, October 13, 1958)

In the previous paper (1) the author reported hyperglycemia and decline of reduced glutathione in blood following administration of sodium cholate.

In 1932, Tate (2) (3) observed a damage of pancreatic islets of rabbits in the experimental jaundice caused by the ligation of bile duct. At the initial stage of the jaundice, the pancreatic islet cells showed an enlargement and other histologic signs exhibiting its increased function, whereas 8 days after the ligation the cells showed the figure of reduced function such as deformation, reduced intensity to staining and other cell-destructive figures. In 1934, Tomozawa (4) reported that the islet of dog pancreas were damaged in the same character as observed by Tate by the injection of bile or of cholate in a dose of 300 mg. per kg. of body weight. However, they described nothing about diabetic symptoms. Other experimental studies on the biochemical effects of cholate have appeared in earlier paper. Hypercholesteremia (5-9), increase of inorganic phosphorus in blood (10), decrease of lactate formation (11, 12), resulting of low R.Q. (13), increased excretion of urinary nitrogen and urinary sulfate (14), increased catabolism of body protein (16, 17), accumulation of uric acid in blood and tissues (18, 19), and activation of glucose-6-phosphatase (15) are the results obtained by the experiments *in vivo* and *in vitro*.

All of these findings are with high resemblance to those observed in human diabetes or experimental one.

In this paper, diabetogenic action of sodium cholate will be described.

METHODS

Blood sugar was determined by Roe's anthrone method (20).

Reducing sugar in urine was detected by the conventional method of Benedict, Nylander and Trommer. For the identification of the sugar, the urine collected was subjected to paperchromatography in solvent systems consisting of butanol-acetic acid-water: 4-1-5 and 2-1-1. On the filter papers carbohydrates were detected with aniline phthalate and anicidin hydrochloride in the usual way.

Urinary pentose was tested by orcinol reagent.

Urinary acetone bodies were examined by nitroprusside reagent.

For identification of xanthurenic acid in urines, the urines were condensed, extracted and subjected to paper chromatography by the method of K o t a k e (21). A part of authentic samples of xanthurenic acid was kindly provided from K o t a k e's institute, and another part was prepared from urine of rats fed with tryptophan and butyrate according to K o t a k e (22).

RESULTS

Male adult rabbits weighing 2.0 to 2.5 kg. were fasted for 40 to 45 hours prior to the experiments. Sodium cholate was prepared as 5 per cent solution in isotonic saline or 25 per cent glucose solution to avoid severe damage of venous vein selected for injection. The solution was prepared freshly in each experiment. Thus prepared solution was administered in the doses of 500 to 800 mg. per kg. of body weight with expence of over 60 minutes for injection. More rapid injection caused prompt death of the animals.

Nineteen rabbits were used in this experiment. All of the animals developed intensive hyperglycemia and hemoglobinuria following the injection of sodium cholate with both of the preparations dissolved in glucose solution and isotonic saline. Most of the rabbits died accompanied by convulsion within 48 hours after the injection, but 5 rabbits illustrated in the Figure survived for several days. In all of the survived cases, secondary

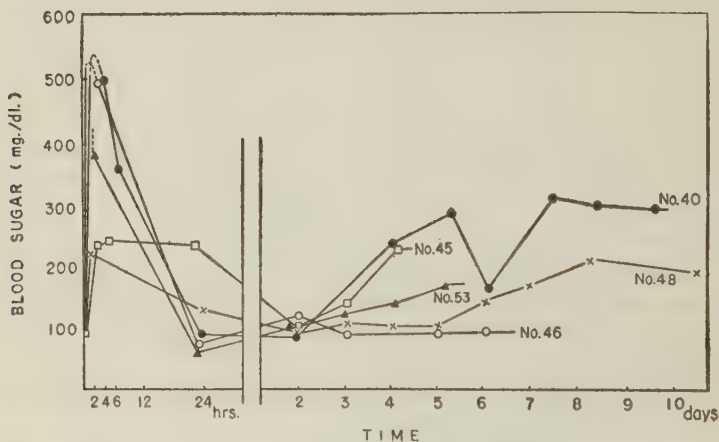


FIG. 1. Curves of the hyperglycemia following the administration of sodium cholate. In the case of No. 45, No. 48 sodium cholate was dissolved in isotonic saline, in the case of No. 46 in 5 per cent glucose and in the cases of No. 40, No. 53 in 25 per cent glucose.

Doses; 600 mg./kg. by No. 40, 750 mg./kg. by No. 45 500 mg./kg. by No. 46, 650 mg./kg. by No. 48 and 550 mg./kg. by No. 53.

continuous hyperglycemia and urinary excretion of large amounts of glucose were observed. In a typical case, No. 40, the initial increase of blood sugar continued for about 24 hours, thereafter the blood sugar returned to its normal level. The second elevation of blood sugar started about 72 hours

after the injection, and the chronic hyperglycemia followed. Accompanied by the second hyperglycemia in each cases glucose was excreted in urines, and the urines gave positive reaction for acetone bodies. In the case of No.

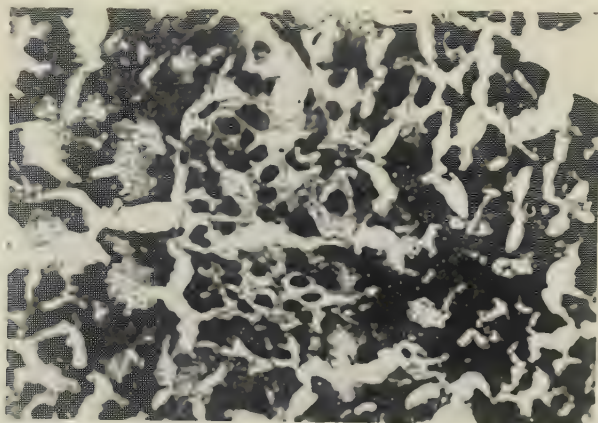


FIG. 2. The pancreatic islet of a normal rabbit.
(Haematoxyrin-Eosin staining)

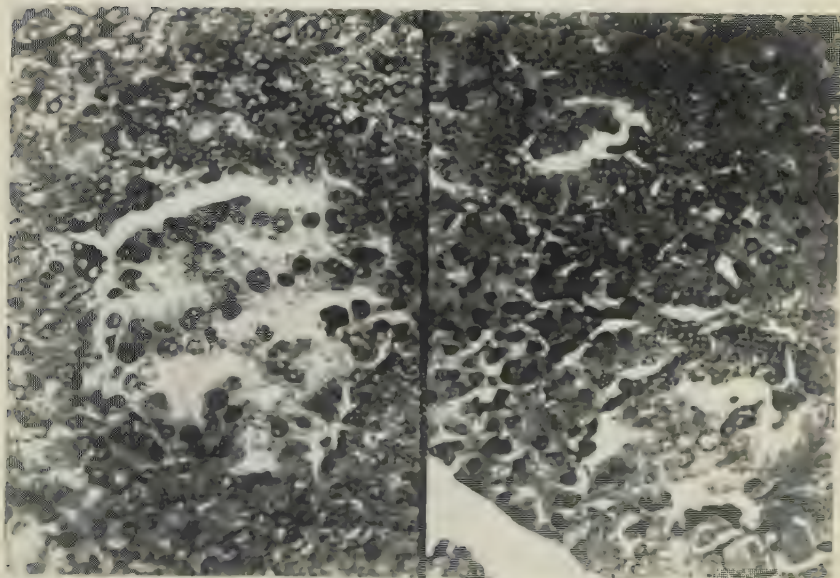


FIG. 3. The damaged pancreatic islets following the injection of sodium cholate.
(Haematoxyrin-Eosin staining)

4b urinary glucose and urinary acetone bodies were observed in spite of absence of hyperglycemia.

Orcinol reaction of the urines was always negative.

The presence of glucose in the urines was ascertained paperchromatographically, but no appreciable amount of free glucuronic acid was detected in the urines.

A trace amount of xanthurenic acid was detected paper chromatographically in the urines.

The animals weakened generally, spoiled their appetit and lost their body weight day by day. All the animals died with convulsion or comatous condition within 10 days after the injection.

The pancreas' were removed after the animals died, and submitted to histological examination. The pancreatic islets were damaged as illustrated in the Figs. with hydropic or pycnotic degeneration of the cells, reduced intensity to staining, confluence of cytoplasm and partial cell-destruction, etc.

DISCUSSION

The glucose contained in the injected cholate solution may cause extreme hyperglycemia just after the injection, but it was shown that hyperglycemia due to single administration of such an amount of glucose, without cholate, was quite temporary, the blood sugar level returning to normal within about 12 hours after administration of glucose. The cholate solution dissolved in isotonic saline also gave an initial hyperglycemia as revealed in the previous paper and in this experiment.

A meaning of xanthurenic acid detected in the urines is obscure.

In the next paper, on the dietary effect about hyperglycemic and diabetogenic action of sodium cholate will be reported, and a possible relation to human diabetes will be discussed.

SUMMARY

The dose of 500 to 800 mg. per kg. of body weight of sodium cholate were administered to 19 rabbits fasted for 40 to 45 hours prior to the experiments. Fourteen rabbits died within 48 hours after the administration, but all of the remaining 5 rabbits became diabetic.

Histological observation revealed the damage of the islets of the pancreas'.

The author wishes to express his thanks to Prof. H. Yoshikawa, Dr. M. Nakao of the Department for their support and encouragement, to Dr. N. Mukai for his aid for histological observation and to members of Kotake's institute for kindly supply of xanthurenic acid.

REFERENCES

- (1) Yanagisawa, I., *J. Biochem.*, **45**, 951 (1958)
- (2) Tate, Y., *Okayama Igakkai Zasshi*, **44**, 2671 (1932)
- (3) Tate, Y., *Okayama Igakkai Zasshi*, **44**, 2851 (1932)
- (4) Tomozawa, T., *Okayama Igakkai Zasshi*, **46**, 1029 (1934)
- (5) Friedman, M., and Byers, S. O., *Proc. Soc. Exptl. Biol. Med.*, **78**, 528 (1951)

- (6) Friedman, M., and Byers, S. O., *Am. J. Physiol.*, **168**, 292, 298 (1952)
- (7) Byers, S. O., and Friedman, M., *J. Exptl. Med.*, **95**, 19 (1952)
- (8) Friedman, M., Byers, S. O., and Roseman, R. H., *Science*, **115**, 313 (1952)
- (9) Siperstein, M. D., Nichols, C. W. Jr., and Chaikoff, I. L., *Science*, **117**, 386 (1953)
- (10) Nakano, T., *Hiroshima Med. Assoc.*, **3**, 185 (1950)
- (11) Teraoka, M., *Biochem. Z.*, **249**, 118 (1932)
- (12) Uraki, Z., *J. Biochem.*, **18**, 207 (1933)
- (13) Hatakeyama, T., *J. Biochem.*, **11**, 273 (1930)
- (14) Kaziro, K., *J. Biochem.*, **7**, 293 (1927)
- (15) Ashmor, J., and Hasting, A. B., transmitted by Knox, W. E. *et al.* in *Physiol. Rev.*, **36**, 175 (1956)
- (16) Karasawa, R., *J. Biochem.*, **6**, 139 (1926)
- (17) Karasawa, R., *J. Biochem.*, **7**, 145 (1927)
- (18) Takeuchi, N., *Okayama Igakkai Zasshi*, **49**, 2133 (1937)
- (19) Takeuchi, N., *Okayama Igakkai Zasshi*, **49**, 1977 (1937)
- (20) Roe, J. H., *J. Biol. Chem.*, **212**, 335 (1955)
- (21) Kotake, Y., Transactions of the Conference on Endocrinology, Tokyo University, **6**, 1 (1955)
- (22) Kotake, Y., and Inada, T., *J. Biochem.*, **40**, 287 (1953)

METABOLIC STUDIES OF BILE ACIDS
XX. '3 β -HYDROXYSTEROL DEHYDROGENASE'
IN RAT LIVER

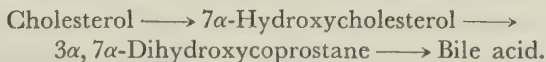
I. ACTIVITY AND FRACTIONATION OF RAT LIVER
HOMOGENATE*

By KAZUMI YAMASAKI, FUMIO NODA AND
KYUTARO SHIMIZU

(From the Department of Biochemistry, Tottori University
School of Medicine, Yonago)

(Received for publication, October 13, 1958)

Recent studies (1) on the conversion of cholesterol to bile acids in the rat have proved that the nuclear transformation of cholesterol molecule resulting in 3 α , 7 α -dihydroxycoprostanane precedes the degradation of the side chain. Lindstedt (2) has lately made an interesting observation that 7 α -hydroxycholesterol is transformed into cholic acid in the rat. The following scheme of the bile acid formation has become highly probable:



Of the above scheme the conversion of 7 α -hydroxycholesterol to 3 α , 7 α -dihydroxycoprostanane includes inevitably a step of the epimerization of 3 β -hydroxyl to 3 α -hydroxyl group with simultaneous saturation of the double bond to give the coprostanane derivative. Any information, however, to picture such a transformation is still lacking, and we have studied the metabolism of 7 α -hydroxycholesterol *in vitro*, and found that in the presence of DPN**, rat liver homogenate oxidizes the sterol to the postulated metabolite, 7 α -hydroxy-3-keto- Δ^4 -cholestene. The activity is detectable, among the several fractions of the homogenate, mainly in the fraction of 30 to 65 per cent ammonium sulfate saturation of the supernatant fluid.

EXPERIMENTAL

Material—7 α -Hydroxycholesterol was prepared according to Fieser *et al.* (3). DPN⁺ was obtained from the Nutritional Biochemicals Corporation. Solvase, obtained from the Mochida Pharmaceutical Mfg. Co., was a mixture of Tween 400 and Tween 4,000 (1:1). Bile acids and other steroids were authentic samples which showed the correct melting points given in the literature.

* The present work was aided by Grant in Aid for Scientific Research from the Ministry of Education, to which our gratitude is due.

** DPN⁺: diphosphopyridine nucleotide.

Color Reagent (Lifschütz)—The reagent was a mixture of 0.1 g. of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 10 ml. of conc. H_2SO_4 and 90 ml. of glacial acetic acid (4), and prepared freshly before use.

Determination of 7 α -Hydroxycholesterol—To a 7 α -hydroxycholesterol solution in 1.5 ml. of chloroform was added 3 ml. of the color reagent. The mixture was allowed to stand at room temperature for 20 minutes, and the absorbance of the resulting blue solution was then read photometrically at 610 $\text{m}\mu$ against a blank (the color reagent without the sterol). The calibration curve was drawn, using solutions containing 50 to 300 μg . of 7 α -hydroxycholesterol and was found to be a straight line (Fig. 1).

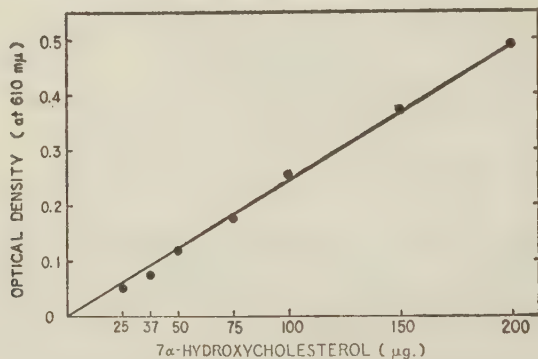


FIG. 1. Calibration curve of 7 α -hydroxycholesterol.

Determination of 7 α -hydroxycholesterol in the homogenate was carried out in the following way: 15 volumes of alcohol-ether mixture (3:1) were added to the homogenate. The mixture was warmed in a water bath for about 30 seconds with occasional shaking and then filtered. The filtrate was evaporated to dryness and the residue was dissolved in 30 ml. of ether. The solution was passed through a column (0.8 $\times$ 2 cm.) of 2 g. of alumina (Brockmann) and the ethereal eluate was discarded. The column was then eluted with 15 ml. of ether-alcohol mixture (9:1). The eluate was evaporated to dryness and the residue was dissolved in 1.5 ml. of chloroform. To the chloroform solution was added 3 ml. of the color reagent and the optical density was read as described above. By these procedures the 7 α -hydroxycholesterol added was recovered within an error of ± 5 per cent.

RESULTS

I. Experiment with Rat Liver Homogenate

Preparation of Homogenate—Rat liver (the Wistar strain) was homogenized in an all-glass homogenizer with three times the volume of one of the following media at 0°. The one of the media was a sucrose solution containing 4.3 g. sucrose (0.13 M) per 100 ml. of 0.08 M Sørensen phosphate buffer (1.01 g. Na_2HPO_4 , 0.14 g. KH_2PO_4 ; pH 7.4), and the other, a potassium chloride solution containing 0.9 g. KCl per 100 ml. of the phosphate buffer.

When 7 α -hydroxycholesterol was incubated with liver homogenate in the presence of DPN^+ , a remarkable disappearance of the sterol was observed, and this was still more marked, when cholate was simultaneously added.

as indicated in Table I. Little or no disappearance was shown in the homogenate without DPN⁺. Such an enhancement of the reaction as shown by the addition of cholate, was also observed when the other bile acids or solvase were used in place of cholate (Table II). These phenomena could be interpreted as meaning that 7 α -hydroxycholesterol, difficult to be dissolved in water, was made more soluble by the addition of the detergent, the metabolism of the sterol being accelerated.

TABLE I
Disappearance of 7 α -Hydroxycholesterol in Rat Liver Homogenate

7 α -Hydroxycholesterol added (μ g.)	No. of Expts.	Nicotinamide, $2 \times 10^{-2} M$	Cholate, $1.5 \times 10^{-3} M$	DPN ⁺ , $2 \times 10^{-3} M$	MgSO ₄ , $2 \times 10^{-3} M$	7 α -Hydroxycholesterol	
						Recovered (μ g.)	Per cent disappearance
200 (100)	3 (4)	+	—	—	—	196 (98)	2 (2)
200	3	+	+	—	—	200	0
200 (100)	3 (3)	+	—	+	—	184 (93)	8 (7)
200 (100)	4 (2)	+	+	+	—	156 (75)	22 (25)
200, 100 (100)	3; 2 (3)	+	+	+	+	133; 66 (71)	33.5; 34 (29)
100 (100)	1 (2)	+	+	—	+	100 (97)	0 (3)
(100)	(5)	+	—	+	+	(80)	(20)
(100)	(2)	+	+	+	—	(75)	(25)

7 α -Hydroxycholesterol was incubated with 3 ml. of the sucrose liver homogenate and the additions noted above in a total volume of 5 ml.; the substrate dissolved in 0.1 ml. of dioxane was added to the homogenate. The incubation was carried out at 38° for 1 hour.

Analogous experiments were carried out with the KCl homogenate (total volume: 4 ml.), and the corresponding figures are shown in parenthesis.

TABLE II
The Influences of Several Detergents upon 7 α -Hydroxycholesterol Metabolism

Detergent	None	Cholate (0.6 mg.)	Chenodeoxycholate (0.6 mg.)	Deoxycholate (0.6 mg.)	Solvase (1 mg.)
Per cent of 7 α -hydroxycholesterol disappeared*	20	34	32	37	34

The reaction mixture, 4 ml., contained 3 ml. of the sucrose liver homogenate, 100 μ g. of 7 α -hydroxycholesterol (in 0.1 ml. of dioxane), $2 \times 10^{-2} M$ of nicotinamide, $2 \times 10^{-3} M$ of DPN⁺, $2 \times 10^{-3} M$ of MgSO₄ and one of the detergents. The mixture was incubated at 38° for 1 hour.

* Mean values for 3 experiments.

II. Fractionation of Rat Liver Homogenate

All the operations were carried out at 0–5°. Chilled rat livers were homogenized with 3 volumes of 0.25 *M* sucrose solution in a glass homogenizer. The homogenate was centrifuged at 600×*g* for 30 minutes, and the precipitate was discarded. The supernatant fluid was centrifuged again at 80,000×*g* for 30 minutes, and the second supernatant fluid was decanted and its volume measured. The precipitate was mixed with the sucrose solution of the equal volume of the supernatant fluid, and stirred. Enzymic

TABLE III
Disappearance of 7 α -Hydroxycholesterol

Fraction	7 α -Hydroxycholesterol	
	Recovered (μ g.)	Per cent disappearance
Supernatant, 3 ml.	31	69
Precipitate, 3 ml.	82	18
Supernatant, 1.5 ml. + precipitate 1.5 ml.	54	46

The incubation mixture, 5 ml., contained 100 μ g. of 7 α -hydroxycholesterol (in 0.1 ml. of dioxane), 1.5 ml. of 0.2 *M* Phosphate buffer (pH 7.2), 2×10^{-3} *M* of MgSO_4 , 1.5×10^{-3} *M* of cholate, 2×10^{-2} *M* of nicotinamide, 0.5 μ M of DPN^+ and one of the homogenate fractions. The mixture was incubated at 38° for 1 hour.

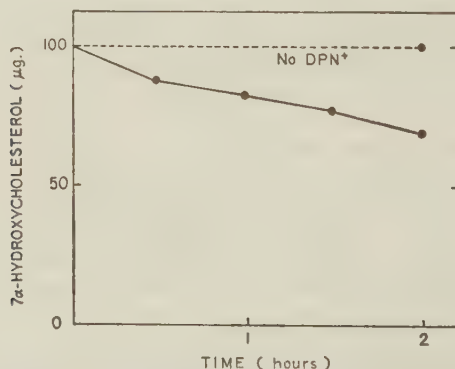


FIG. 2. Rate of disappearance of added 7 α -hydroxycholesterol.

The reaction mixture, 4 ml., contained 1.5 ml. of 0.2 *M* phosphate buffer, 1.5 ml. of the enzyme solution, 2×10^{-3} *M* of DPN^+ , 100 μ g. of 7 α -hydroxycholesterol (in 0.1 ml. of dioxane), 2×10^{-2} *M* of nicotinamide, and 2×10^{-3} *M* of MgSO_4 . The incubation was carried out at 38°.

activities of the two fractions thus obtained, the supernatant and the precipitate, were measured, and the supernatant fraction was found to contain almost all the activity (Table III). This fraction was subjected to further fractionation with ammonium sulfate and the major activity was found in the fraction of 30 to 65 per cent saturation. The fraction, dialyzed, against distilled water as usual, was used as the enzyme solution for the following studies.

Fig. 2 shows that 7 α -hydroxycholesterol disappeared with the time of incubation (at 38°).

TABLE IV
Ammonium Sulfate Fractionation and Enzyme Activity

Per cent saturation of (NH ₄) ₂ SO ₄	7 α -Hydroxycholesterol	
	Recovered (μ g.)	Per cent disappearance
S ₀₋₂₀	98	2
S ₂₀₋₃₀	93	7
S ₃₀₋₆₅	75	25
S ₆₅₋₉₀	100	0
S ₉₀₋₁₀₀	100	0

Each fraction of the ammonium sulfate precipitation was dissolved in 100 ml. of water and dialyzed against distilled water until it was free from sulfate ions; 5 ml. of the incubation mixture contained: 1.5 ml. of 0.2 *M* phosphate (pH 7.2), 100 μ g. of 7 α -hydroxycholesterol, 0.5 μ M of DPN⁺, 2×10^{-2} *M* of nicotinamide, 2×10^{-3} *M* of MgSO₄ and 3 ml. of each fraction noted above. Incubation was carried out at 38° for 1 hour.

TABLE V
Reduction of 2,6-Dichlorophenol Indophenol by Several Steroids in the Thunberg Apparatus

Substrate	2,6-Dichlorophenol indophenol
7 α -Hydroxycholesterol	Decolorized in 1.5 hours
Cholesterol	Decolorized in 1.8 hours
Androsterone	No decolorization within 3 hours
Dehydroepiandrosterone	"
Pregnenolone	"
3 α , 7 α , 12 α -Trihydroxycoprostan	"

The tube contained 1 ml. of 0.005 per cent solution of 2,6-dichlorophenol indophenol and 1 ml. of substrate emulsion. The substrate emulsion was prepared as followed: To the solution containing 100 μ g. of each steroid in 0.1 ml. of dioxane was added 0.9 ml. of hot water. The side arm contained 0.5 ml. of the enzyme solution, 0.1 mole of DPN⁺ in 0.5 ml. of water and 0.5 ml. of 0.2 *M* Tris buffer (pH 7.4). The contents of the tube and the side arm were mixed and incubated at 38°.

III. Incubation in the Thunberg Apparatus

In order to test the dehydrogenase character and substrate specificity of the enzyme solution, several steroids were incubated in the Thunberg apparatus using dichlorophenol indophenol as a hydrogen acceptor (Table V). Reduction of the dye was observed only when 7α -hydroxycholesterol or cholesterol was used as substrate. Androsterone, dehydroepiandrosterone, pregnenolone and $3\alpha, 7\alpha, 12\alpha$ -trihydroxycoprostone showed no reduction within 3 hours.

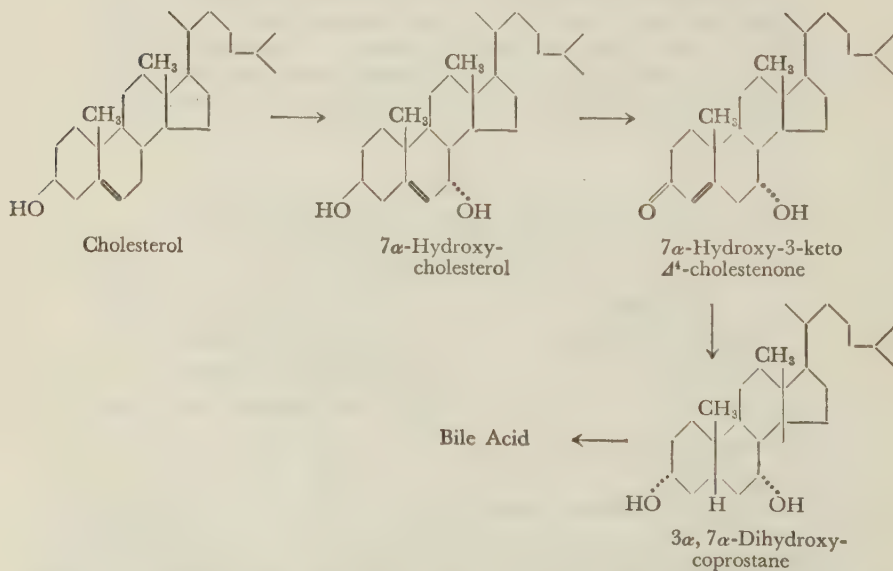
DISCUSSION

From the supernatant fluid of rat liver homogenate we have prepared an active fraction by fractional precipitation with ammonium sulfate, which oxidizes 7α -hydroxycholesterol in the presence of DPN⁺. Cholesterol is also dehydrogenated by this fraction, but either 3α -hydroxysteroids in general or 3β -hydroxysteroids of the C₁₉ and C₂₁ series are not. The active fraction, specific for 3β -hydroxysteroids is apparently different from the bacterial 3β -hydroxysteroid dehydrogenase of Talalay *et al.* (5). We would, therefore, call the enzyme or enzyme system 3β -hydroxysteroid dehydrogenase.

The present demonstration that 7α -hydroxycholesterol, convertible into

DIAGRAM 1.

A Tentative Scheme for Bile Acid Formation from Cholesterol



bile acid *in vivo* (2), is readily oxidized by the enzyme of rat liver, 3β -hydroxysteroid dehydrogenase, strongly indicates that the resulting product, 7α -hydroxy-3-keto- Δ^4 -cholestene, is the intermediate of the epimerization of 3β -hydroxyl group of the sterol. 7α -Hydroxyl group of the sterol might be

a determinant for the epimerization, because Harold *et al.* (6) have shown that Δ^4 -cholestenone is readily converted to cholestanol *in vivo* as well as *in vitro*. The scheme of the bile acid formation presented at the beginning of this paper could now be supplemented as indicated in Diagram 1.

SUMMARY

1. From the rat liver an active fraction, 3 β -hydroxysterol dehydrogenase, was prepared which dehydrogenates 7 α -hydroxycholesterol in the presence of DPN⁺. This fraction was also capable of oxidizing cholesterol, but neither 3 α -hydroxy steroids in general nor 3 β -hydroxy steroids of the C₁₉ and C₂₁ series were affected.

2. A metabolic course of cholesterol to bile acid was discussed.

The authors are indebted to Prof. Dr. L. F. Fieser, Harvard University, for generous gifts of pregnenolone and other steroids. Thanks are due to Miss N. Hatanô for her expert technical assistance during the course of this work.

REFERENCES

- (1) Bergström, S., *Record of the Chemical Progress*, **16**, 63 (1955)
- (2) Lindstedt, S., *Acta Chem. Scand.*, **11**, 417 (1957)
- (3) Fieser, L. F., Fieser, M., and Chakravarti, R. N., *J. Am. Chem. Soc.*, **71**, 2226 (1949)
- (4) Bergström, S., and Wintersteiner, O., *J. Biol. Chem.*, **145**, 327 (1942)
- (5) Talalay, P., and Marcus, P. I., *J. Biol. Chem.*, **218**, 675 (1955)
- (6) Harold, F., Abraham, S., and Chaikoff, I. L., *J. Biol. Chem.*, **221**, 435 (1955); Harold, F. M., Chapman, D. D., and Chaikoff, I. L., *J. Biol. Chem.*, **224**, 609 (1956)

METABOLIC STUDIES OF BILE ACIDS
XXI. '3 β -HYDROXYSTEROL DEHYDROGENASE'
IN RAT LIVER*

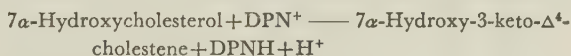
II. PURIFICATION, SPECIFICITY AND INHIBITION

By KAZUMI YAMASAKI, FUMIO NODA AND KYUTARO SHIMIZU

(From the Department of Biochemistry, Tottori University
School of Medicine, Yonago)

(Received for publication, October 13, 1958)

In the preceding communication (1) it is reported that a cell-free preparation of rat liver homogenate is capable of oxidizing 7 α -hydroxy-cholesterol to a proposed metabolite, 7 α -hydroxy-3-keto- Δ^4 -cholestene in the presence of DPN⁺ **, and should be designated as 3 β -hydroxysterol dehydrogenase, different from the bacterial 3 β -hydroxysteroid dehydrogenase (2). The present experiment, carried out with a relatively pure enzyme preparation, deals with certain aspect of substrate and coenzyme specificity of this preparation. The velocities of the reactions catalyzed by this preparation will be considered. The proposed mechanism is the conversion of 7 α -hydroxycholesterol to 7 α -hydroxy-3-keto- Δ^4 -cholestene as follows:



This purified enzyme preparation is capable of oxidizing 7 α -hydroxy-cholesterol and cholesterol, but not the 3 β -hydroxysteroids of the C₁₉ and C₂₁ series, exactly like the crude preparation reported previously.

EXPERIMENTAL

Substrate—Pregnenolone, dehydroepiandrosterone and cholestenone were kindly supplied by Prof. Dr. Fieser, Harvard University, U.S.A. 7 α -Hydroxycholesterol (3), cholestanol (4), and Δ^5 -cholestenone*** were prepared from cholesterol. 3 β -Hydroxy- Δ^4 -cholestene was prepared by reducing cholestenone with LiAlH₄ in ether (5). All steroids were carefully purified by recrystallization until the melting point of each agreed with that given in the literature.

Preparation of Substrate Solution—Because of relative insolubility of steroids in water, a 10 μ g. per ml. solution of each steroid was prepared as follows: One mg. of the steroid

* The present work was aided by the Grant in Aid for Scientific Research from the Ministry of Education, to which our gratitude is due.

** The abbreviations, DPN⁺ and DPNH (oxidized and reduced diphosphopyridine nucleotide, respectively), and TPN⁺ (oxidized triphosphopyridine nucleotide) are used in this report.

*** Prepared according to the private communication of Prof. L. F. Fieser, Harvard University.

was dissolved in 10 ml. of methanol, 1 ml. of which was diluted rapidly with 9 ml. of hot water.

Coenzyme—DPN⁺ and TPN⁺ were obtained from the Nutritional Biochemicals Corporation, U.S.A. DPNH was prepared by the hydrosulfite reduction procedure (6).

Enzyme Assay—The activity of the enzyme solution was calculated from the initial rate of DPN⁺ reduction measured at 340 m μ in a Beckman Model DU spectrophotometer at room temperature. The reactions were carried out in quartz cells having a 1.0 cm. light path and a capacity of about 4 ml.

The reaction velocity was almost linear with time within 10 minutes and an enzyme preparation was defined as having 1 unit of activity if it caused an increase in optical density of 0.001 during the initial 10 minutes after the addition of the enzyme solution to the reaction mixture. Specific activity was defined as units of activity per mg. of protein. Protein was determined by a turbidometric method (7).

Fig. 1 shows the dependence of reaction velocities on enzyme concentrations. Our enzyme preparation shows no DPN⁺ reduction in the absence of substrate. 0.2 ml. of the enzyme solution was used in the following experiments, if not otherwise stated.

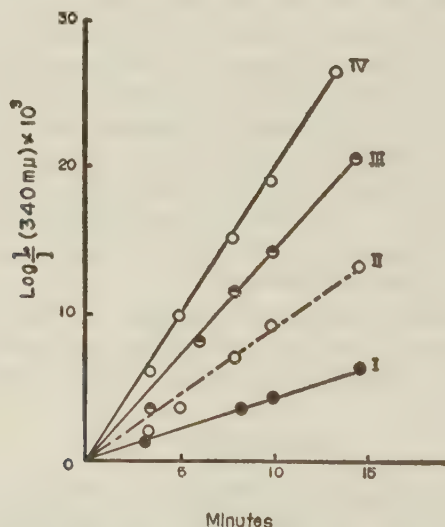


FIG. 1. Dependence of Oxidation Velocities on Amounts of the Enzyme Solution.

The complete system (3.2 ml.) contained 200 μ M of phosphate buffer, pH 7.4, 0.25 μ M of DPN⁺, 10 μ g. of sterol and 0.1, 0.15, 0.2 and 0.5 ml. of the enzyme solution (Curve I-IV). The measurements were made at 25° and at 340 m μ in quartz cells having a 1.0 cm. light path. Control cells contained all components except sterol.

RESULTS

Purification of Enzyme Solution—Two rats were killed by a shary blow on the head and their livers were rapidly excised and chilled. They were then weighed and homogenized in an all-glass homogenizer with 3 volumes of

cold 0.25 *M* sucrose. Subsequent operations were carried out at 0–5°. The homogenate was centrifuged at 80,000 $\times g$ for 30 minutes and the precipitate was discarded. To the supernatant fluid was added a saturated ammonium sulfate solution, neutralized to pH 7 with NH₄OH, until there was obtained an ammonium sulfate solution of 30 per cent saturation. After 10 minutes the solution was centrifuged at 10,000 $\times g$ for 10 minutes. The precipitate was discarded, and the supernatant fluid was mixed with an equal volume of saturated ammonium sulfate solution. After 10 minutes the mixture was again centrifuged, and the precipitate dissolved in cold distilled water was dialyzed against distilled water until it was free from sulfate ions. Any precipitate which was formed during the dialysis was centrifuged.

One tenth volume of 0.2 *M* phosphate buffer, pH 6.2 was added to the dialyzed solution, which was centrifuged after 10 minutes and the precipitate was discarded. The supernatant fluid was treated with alumina C₇ as follows: To this fluid was added one tenth volume of the gel suspension, which had been aged for one month and contained 1 mg. of solid per ml. and the mixture was stirred for 10 minutes and then centrifuged. The supernatant fluid, adjusted to pH 7.4, was used as the enzyme solution. A summary of the purification procedure appears in Table I.

TABLE I
Summary of Enzyme Purification

Fraction	Volume, ml.	Unit	Specific activity
(NH ₄) ₂ SO ₄ ppt.	14	308	3.6
Supernatant of pH 6.2 ppt.	18	432	9
Supernatant of alumina C ₇ .	20	360	11

Stability of the Enzyme Solution—The enzyme solution was stable for at least 2 weeks at –10° without any loss of activity. It was found that the enzyme preparation lost its activity when it was preincubated in one per cent solution of methanol for 30 minutes at room temperature (Fig. 2).

Coenzyme Specificity—The enzyme solution reacted with both DPN⁺ and TPN⁺, and the latter had the same activity as the former. The relationship of reaction velocities to concentrations of each of the coenzymes is shown in Fig. 3.

Effect of pH—The optimum pH of the DPN⁺ reduction by 7 α -hydroxycholesterol was found between 7.4 and 8.5 in either Tris or phosphate buffer (Fig. 4).

Inhibition by Sulfhydryl Reagents—The presence of sulfhydryl groups in the enzyme system was suggested by inhibitory effect of both heavy metal ions and iodoacetate (Table II).

Substrate Specificity—As shown in Table III, the enzyme preparation was capable of oxidizing 3 β -hydroxysterols (C₂₇), regardless of the configuration

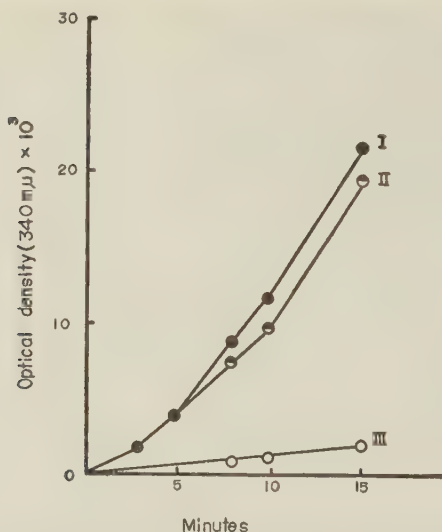


FIG. 2. Inactivation of the enzyme in methanol solution.

The cells contained 200 μ M of phosphate buffer, pH 7.4, 0.25 μ M of DPN⁺, 1 ml. of 7 α -hydroxycholesterol solution and 0.25 ml. of the enzyme solution.

Curve I: All components of the system were simultaneously mixed and the reaction was started.

Curve II: The enzyme solution was pre-incubated for 30 minutes at room temperature with the buffer and DPN⁺, and the reaction was started by addition of the substrate and 0.1 ml. of methanol.

Curve III: The enzyme solution was pre-incubated for 30 minutes at room temperature with the buffer, DPN⁺ and 0.1 ml. of methanol, and the reaction was started by addition of the substrate.

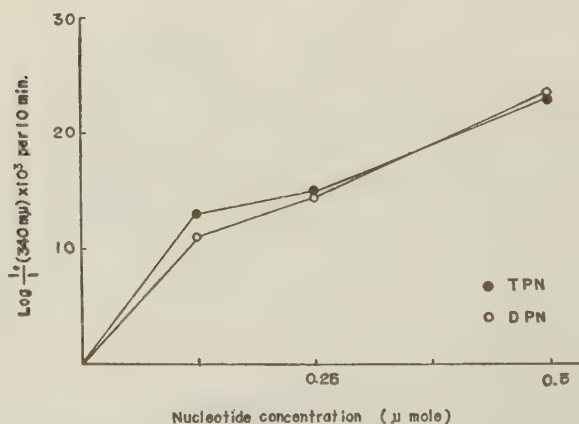


FIG. 3. Effect of the concentration of coenzyme on the reaction velocity.

The cells contained the enzyme solution, 10 μ g. of 7 α -hydroxycholesterol, 200 μ M of phosphate buffer, pH 7.4 and different amounts of DPN⁺ or TPN⁺ in a total volume of 3.2 ml.

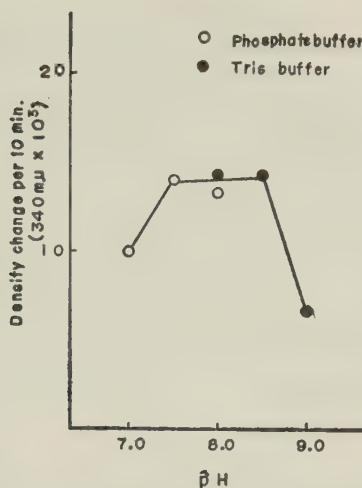


FIG. 4. Effect of pH on the reaction velocity.

The cells contained 10 μ g. of 7 α -hydroxycholesterol, 0.25 μ M of DPN⁺, 200 μ M of phosphate buffer, and the enzyme solution in a total volume of 3.2 ml.

TABLE II
Inhibition by Sulfhydryl Reagents

Inhibitor	Per cent inhibition
Cu ²⁺ , 10 ⁻⁴ M	70
Iodoacetate, 10 ⁻⁴ M	100
Iodoacetate, 2 $\times$ 10 ⁻⁵ M	80
Iodoacetate, 10 ⁻⁵ M	60
Iodoacetate, 5 $\times$ 10 ⁻⁶ M	20

The cells contained 0.25 μ M of DPN⁺, 100 μ M of phosphate buffer, pH 7.4, 10 μ g. of 7 α -hydroxycholesterol, the enzyme solution, and inhibitor to a volume of 3.0 ml.

of the A-B ring junction (5 α - or 5 β -series; Δ^4 - or Δ^5 -series) and 3 β - Δ^5 -hydroxycholonic acid (C₂₄). Neither the C₁₉ nor C₂₁ 3 β -hydroxysteroids, however, were oxidized with a notable exception of Δ^4 -androstene-3 β , 17 β -diol*. None of the 3 α -hydroxysteroids so far tested was dehydrogenated by our preparation, either.

Reversibility of the Enzyme Action—The enzyme preparation also catalyzed the reduction of 3-keto sterols in the presence of DPNH. Fig. 5 shows the enzymic reduction of Δ^4 -cholestenone and Δ^5 -cholestenone.

Identification of the Product formed—That 3 β -hydroxy- Δ^5 -sterols were oxidized to Δ^4 -3-keto-compounds, was ascertained by observing increases in

* It is highly probable that the 17 β -hydroxyl group of this diol was dehydrogenated, because testosterone was likewise oxidized.

TABLE III
Relative Rates of Enzymic Oxidation of 3-Hydroxysteroids

Substrate	Relative rate of oxidation
7 α -Hydroxycholesterol	100
Cholesterol	60
7-Oxocholesterol	80
Δ^4 -Cholestene-3 β -ol	80
Cholestanol	90
Coprostanol	25
3 α , 7 α , 12 α -Trihydroxycoprostanol	0
Pregnenolone	0
Dehydroepiandrosterone	0
Δ^4 -Androstene-3 β , 17 β -diol	175
Testosterone	100
3 β -Hydroxy- Δ^5 -cholenate*	190
Lithocholate*	0
Cholate*	0

The cells contained 0.25 μ M of DPN⁺, 10 μ g. of substrate, 200 μ M of phosphate buffer, pH 7.4 and enzyme solution to a volume of 3.2 ml.

* Each bile acid was dissolved in an equivalent amount of NaOH solution.

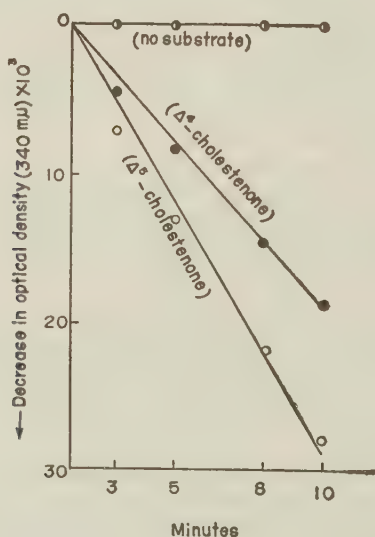


FIG. 5. Reduction of Ketosterols.

Cuvettes contained 100 μ M of phosphate buffer, pH 7.4, 0.12 μ M of DPNH, 10 μ g. of substrate, and 0.5 ml. of the enzyme solution in a total volume of 3.0 ml.

optical density at 240 $m\mu$. The measurements of the reaction product were made as follows: 0.5 ml of the enzyme solution, 10 μ g. of substrate, 0.25 μ M of DPN⁺ and 200 μ M of phosphate buffer, pH 7.4, were mixed and incubated at room temperature for 10~20 minutes. The reaction mixture (3 ml.) was pipetted into 5 ml. of chloroform in a glass-stoppered tube, shaken vigorously for about 30 seconds and centrifuged. The aqueous layer and the coagulated protein were removed. The same procedure of extraction was repeated with the reaction mixture of zero time. The optical density at 240 $m\mu$ of both chloroform extracts was read. From the difference between the readings was calculated the amount of Δ^4 -3-keto compound then formed.

Another series of the reactions was carried out in a quartz cell of the spectrophotometer and after 10~20 minutes, increase in optical density at 340 $m\mu$ was read against a control containing the components of the system except sterol.

TABLE IV

DPN⁺ Reduction during the Oxidation of Cholesterol and 7 α -Hydroxycholesterol

Exp. No.	Substrate	DPN ⁺ reduced (μ M $\times 10^{-4}$)	Δ^4 -3-Keto grouping formed (μ M $\times 10^{-4}$)
1	Cholesterol	115	80
2	"	46	50
3	"	25	32
4	7 α -Hydroxy-cholesterol	100	90
5	"	75	74
6	"	65	64

The determination of DPNH and Δ^4 -3-keto grouping in the reaction mixtures was conducted as described in the text.

As indicated in Table IV, the increase in optical density at 340 $m\mu$ corresponded fairly well to that at 240 $m\mu$, and approximately 1 mole. of DPN⁺ was reduced per mole of the substrate oxidized. Here were taken the value of 6.22×10^6 cm²/mole (β) and the approximate value of 10^4 cm²/mole (γ) as the molecular extinction coefficient of DPNH at 340 $m\mu$ and that of Δ^4 -3-keto-sterol at 240 $m\mu$, respectively.

Among the sterols tested here 7-oxocholesterol expectedly presented an exception that an increase in optical density at 340 $m\mu$, but not at 240 $m\mu$, was observed when it was incubated.

In order to identify the metabolic product of cholesterol, a mixture of 1 ml. of cholesterol emulsion (1 per cent serum albumin solution), 1 mg. of DPN⁺, 800 μ M of phosphate buffer and 3 ml. of the enzyme solution was incubated at 37° for 1 hour. The mixture was extracted with chloroform and the extract was subjected to paper chromatography according to Hansen and Dam (10). The product was located on the paper by means of α -naphthylamine phthalate spray for fructose detection (11). Only

a single metabolite was detectable, which had a mobility ($R_f=0.75$) identical with that of cholestenone (Fig. 6).

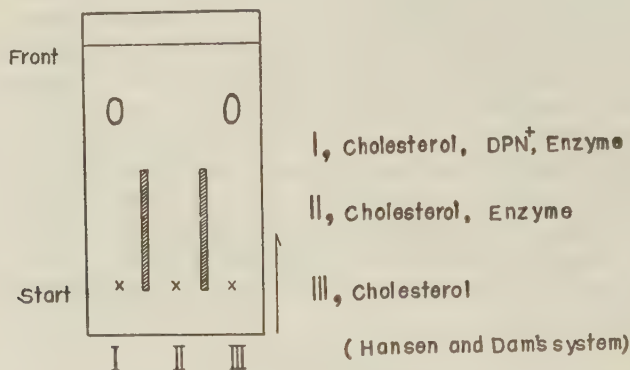


FIG. 6. Chromatogram of the reaction product of cholesterol.

DISCUSSION

In this report the authors have described the presence of an enzyme or enzyme system in rat liver which catalyzes oxidation of 3β -hydroxysterols to 3-ketosterols. Our enzyme preparation, 3β -hydroxysterol dehydrogenase, catalyzes oxidation of 3β -hydroxysterols and 3β - Δ^5 -hydroxycholeonic acid, while 3β -hydroxysteroids of the C_{19} and C_{21} series are not metabolized by this preparation. Accordingly the enzyme is quite different in its properties from the bacterial 3β -hydroxysteroid dehydrogenase of Talalay *et al.* (2). It is interesting to note, however, that 17β -hydroxysteroids, androstene- 3β , 17β -diol and testosterone are oxidized by our preparation, just as the bacterial β enzyme.

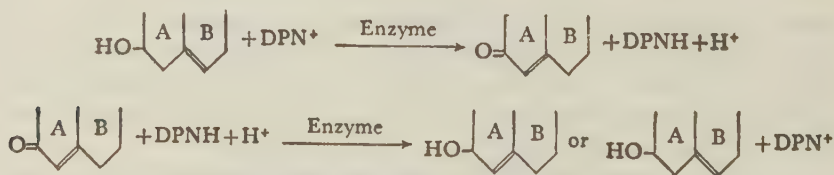


FIG. 7. The proposed mechanism of 3β -Hydroxysterol dehydrogenase.

The enzyme apparently oxidizes 3β -hydroxy- Δ^5 -sterols to their corresponding 3-keto- Δ^4 -sterols (Fig. 7), and the reaction is probably a reversible one, though it is uncertain whether the reduction products of 3-keto- Δ^4 -sterols are 3β -hydroxy- Δ^4 - or 3β -hydroxy- Δ^5 -compounds. Since 3β -hydroxysterols dehydrogenase has now been found in rat liver, it might be unnecessary to assume that when cholesterol is metabolized to bile acid, shift of the double bond precedes the oxidation of 3β -hydroxyl to 3-keto group, as

suggested by Ungar *et al.* (12).

SUMMARY

1. A partial purification of 3 β -hydroxysterol dehydrogenase from rat liver has been described.

2. The enzyme preparation catalyzed the reversible oxidation of 3 β -hydroxysterols, regardless of the configuration of the A-B ring junction.

3. DPN⁺ and TPN⁺ functioned here equally well as a hydrogen acceptor.

4. The enzyme preparation was inhibited by some sulfhydryl reagents.

The authors are indebted to Prof. Dr. L.F. Fieser, Harvard University, for generous gifts of pregnenolone and other steroids. Thanks are due to Miss N. Hatano for her expert technical assistance during the course of this work.

REFERENCES

- (1) Yamasaki, K., Noda, F., and Shimizu, K., *J. Biochem.*, **46**, 739 (1959)
- (2) Talalay, P., and Marcus, P. I., *J. Biol. Chem.*, **218**, 675, (1955)
- (3) Fieser, L. F., Fieser, M., and Chakravarti, R. N., *J. Am. Chem. Soc.*, **71**, 2226 (1949)
- (4) Willstätter, P., and Meyer, F. W., *Ber.*, **41**, 2199 (1908)
- (5) Fieser, L. F., and Fieser, M., Natural Products related to Phenanthrene, Reinhold Publishing Corporation III Ed., p. 239 (1949)
- (6) Umbreit, W. W., Burris, R. G., and Stauffer J. F., Manometric Technique and Tissue Metabolism, Burgess Publishing Co., p. 217 (1951)
- (7) Stadtman, E. R., Novelli, G. D., and Lipman, F., *J. Biol. Chem.*, **191**, 365 (1951)
- (8) Horecker, B. L., and Kornberg, A., *J. Biol. Chem.*, **175**, 385 (1948)
- (9) Fieser, L. F., and Fieser, M., Natural Products related to Phenanthrene, Reinhold Publishing Corporation III Ed., p. 191, (1949)
- (10) Hansen, P. W., and Dam, H., *Acta Chem. Scand.*, **11**, 1658 (1957)
- (11) Bodanszky, A., and Collonitsch, J., *Nature*, **173**, 729 (1955)
- (12) Ungar, F., Gut, M., and Dorfman, R. I., *J. Biol. Chem.*, **224**, 191 (1957)

STUDIES ON RIBONUCLEASES IN TAKADIASTASE

III. PURIFICATION AND PROPERTIES OF RIBONUCLEASE T₂*

By MARIKO NAOI-TADA, KIMIKO SATO-ASANO AND FUJIO EGAMI

(From the Department of Chemistry, Faculty of Science,
Nagoya University, Nagoya)

(Received for publication, October 18, 1958)

In the series on this investigation we have already reported the existence of RNase** T₁ and RNase T₂, and the purification and properties of the former (1-4). In this paper purification and properties of RNase T₂ will be described.

EXPERIMENTAL

Materials and methods were same as described in the previous papers (1, 3).

Purification of RNase T₂

As RNase T₂ has its pH optimum at 4.5, while RNase T₁ has it at 7.5 (1), the difference of optimal pH was used as the indicator of RNase T₂ during the purification. By the preliminary experiments it was shown that RNase T₂ precipitated at lower saturation of ammonium sulfate than RNase T₁ and RNase T₂ was also stable for heating at pH 2.5 in the crude extract as RNase T₁. In order to obtain RNase T₂ free from RNase T₁ the purification was carried out as follows.

Heat Treatment—100 g. of "Takadiastase Sankyo" was extracted with 500 ml. of distilled water. The extract was acidified to pH 2.5 with HCl and heated to 80° for 2 minutes in a boiling water bath. After cooling in an ice-bath the precipitate was removed by filtration. The supernatant was adjusted to pH 6.0 with ammonium hydroxide and the precipitate formed was removed off.

Ammonium Sulfate Fractionation—The filtrate was brought to 0.45 saturation of ammonium sulfate by the addition of solid ammonium sulfate. The precipitate was collected by filtration and extracted with 0.1 M citrate buffer (pH 6.0). The extract was brought to 0.65 saturation of ammonium sulfate and refiltered. The precipitate was dissolved in the buffer and dialysed against distilled water in a cold room.

* Aided by a grant from the Scientific Research Fund of the Ministry of Education.

** Abbreviations: ribonuclease, RNase; ribonucleic acid, RNA; phosphomonoesterase, 1'Mase; 2'-adenylic acid, 2'-Ap; 3'-adenylic acid, 3'-Ap; 2'-guanylic acid, 2'-Gp; 3'-guanylic acid, 3'-Gp; uridylic acid, Up; cytidylic acid, Cp.

Adsorption on Calcium Phosphate Gel—The dialysed solution was adjusted to pH 5.6 with acetate buffer (Final concentration 0.02 *M*) and suspension of calcium phosphate gel (5) was added dropwise. The elution was carried out in two stages; the first eluate with 0.05 *M* Na₂HPO₄ containing the RNase T₁ activity was removed, RNase T₂ was then eluted with 0.2 *M* Na₂HPO₄ twice, and these eluates were combined and dialysed.

TABLE I
Purification of RNase T₂

Steps	Activity at pH 7.5
	Activity at pH 4.5
Crude extract	1.2
Heat treatment	1.6
(NH ₄) ₂ SO ₄ fractionation	0.4
Ca ₃ (PO ₄) ₂ gel	0.4
DEAE-cellulose column	0.2

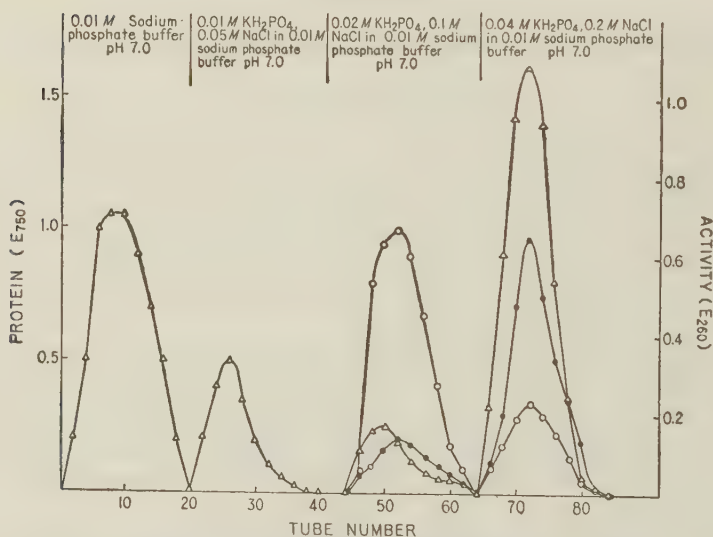


FIG. 1. Chromatogram of RNase T₂ on DEAE-cellulose column. It is the curve of first chromatography. Flow rate was 3 ml./hour. One fraction was 2 ml. For measurement of activity and protein, 0.1 ml. and 1.0 ml. was used respectively. —Δ—, protein; —○—, activity at pH 4.5; —●—, activity at pH 7.5.

Fractionation on DEAE-cellulose Column—Dialysed solution was adsorbed on diethylaminoethyl-cellulose (DEAE-cellulose, prepared by the method of Peterson and Sober (6) from Whatman cellulose powder, and nitrogen content was 0.88 meq./g. of cellulose) column in the 0.01 *M* phosphate buffer (pH 7.0). RNase T₂ was eluted from the column with KH₂PO₄-NaCl

solution. This chromatography was repeated twice. The chromatographic pattern is shown in Fig. 1. The results of the purification is shown in Table I and from 100 g. of Takadiastase about 1 mg. of RNase T₂ was obtained.

Homogeneity of RNase T₂—The homogeneity of the most purified preparation was tested by zone-electrophoresis in veronal buffer (pH 8.5, $\mu=0.1$) for 7 hours. The result is shown in Fig. 2. RNase activity showed only one peak at the same position of the peak of the protein measured by Lowly *et al.* This preparation seems to be free from RNase T₁, for RNase T₁ migrates about 7–8 cm. in the same condition.

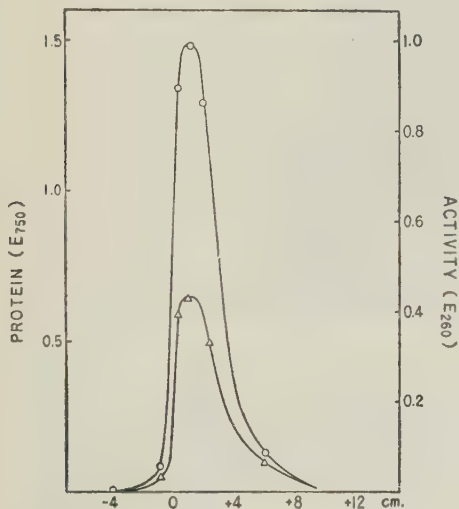


FIG. 2. Zone-electrophoretic pattern of purified RNase T₂ on starch column; in veronal buffer, $\mu=0.1$, pH 8.5; at a field strength of 240 volts/40 cm.; 7 hours electrophoresis. —○—, activity at pH 4.5; —△—, protein.

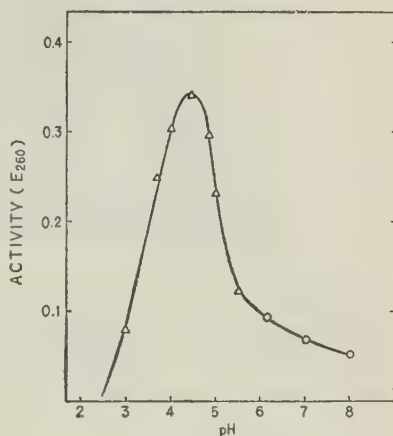


FIG. 3. pH-Activity curve of RNase T₂. The final concentration of buffers were 0.05 M. —△—, acetate buffer; —○—, phosphate buffer.

Properties of RNase T₂

Stability—After heating to 80° for 5 minutes at pH 6.0 activity did not change. The most purified preparation was tested in storage under various pH for a week in a cold room. It was most stable at pH 5.0 and under the condition 25 per cent activity was lost.

Effect of pH—The influence of pH on enzyme activity is shown in Fig. 3. The activity at pH 7.5, optimum of RNase T₁, was one fifth of that at maximum.

Inhibition and Activation—The RNase T₂ was inhibited by some metal ions, *e.g.* Cu⁺⁺ inhibited RNase T₂ completely at 10⁻³ M, and it was activated by EDTA. Therefore in the following experiments EDTA was added to the reaction mixture. Monoiodoacetate and *p*-chloromercuribenzoate

activated the enzyme.

TABLE II
Activators and Inhibitors

Reagents	Final concentration (-log <i>M</i>)	Activation (%)
MgCl ₂	1	-25
	3	0
CaCl ₂	2	-20
NaCl	1	-10
ZnSO ₄	3	0
CuSO ₄	3	-95
NaF	1	10
NaN ₃	2	0
ICH ₂ COONa	2	20
PCMB	4	25
EDTA	2	40
	3	20

Activity was measured without EDTA, at pH 4.5 using the most purified enzyme.

Specificity—To find out the specificity of RNase T₂, digestion of yeast RNA was pursued by measuring the uranium reagent soluble phosphate and paper chromatography. At various intervals the uranium reagent soluble organic phosphate was measured, it increased almost linearly and reached equilibrium after 20 hours at 30 per cent of total phosphate. RNase T₂ could hydrolyse the 'core' of RNase I, even faster than RNA, and 50 per cent of total phosphate was converted to uranium reagent soluble.

Paper chromatogram of the hydrolysate was shown in Fig. 4. Spot 1' (between 1 and 2) appeared at first and increased, but other spots did not appear till 24 hours digestion. The *R_f* value of the spot was the same as that of guanosine-2', 3'-cyclic phosphate (3), but the spot had no characteristic fluorescence of guanine derivatives. The ultraviolet absorption spectrum of the eluate from the spot with 0.01 *N* HCl is shown in Fig. 5. As shown by the absorption curve the spot corresponded to neither of known mononucleotides. So the spot seems to be that of some dinucleotide.

To determine the internucleotide linkage which RNase T₂ attacks specifically, mononucleotide and terminal nucleotide of oligonucleotides were detected. The reaction mixtures same as in Fig. 4 were incubated for various time intervals, and to the mixtures HCl was added to stop the reaction and to hydrolyse the 2', 3'-cyclic phosphate, if produced, to 2'- and 3'-phosphate. After HCl hydrolysis over night at 2°, the mixture was adjusted to pH 5.0 and digested by phosphomonoesterase (2, 3) for 2 hours at 37°. Finally all oligonucleotides were hydrolysed by 1 *N* NaOH for 24

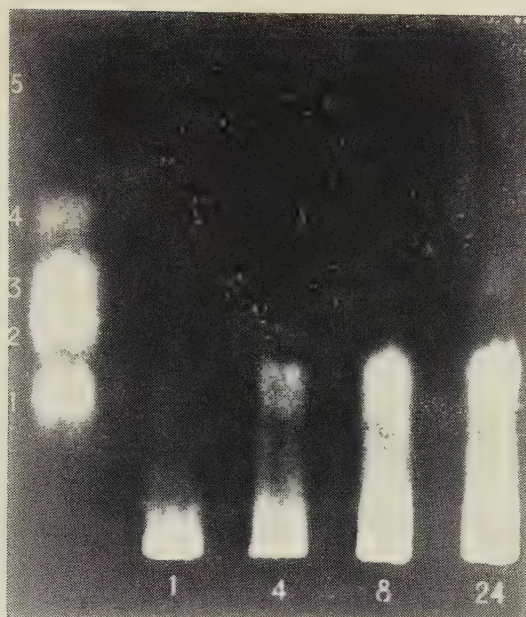


FIG. 4. Paper chromatogram of the digestion products of yeast RNA by RNase T₂. Spots are; (1), 3'-Ap; (2), 2'-Ap; (3), 3'-Gp, (4), 2'-Gp; (5), pyrimidine nucleotides. Solvent; saturated ammonium sulfate:water:isopropanol (79:19:2) (7). 0.5 ml. of the digestion mixtures containing 10 mg. yeast RNA, 60 units of RNase T₂, 10⁻³ M EDTA, and 0.1 M acetate buffer (pH 4.5), were incubated at 37°, for 1, 4, 8 and 24 hours, respectively.

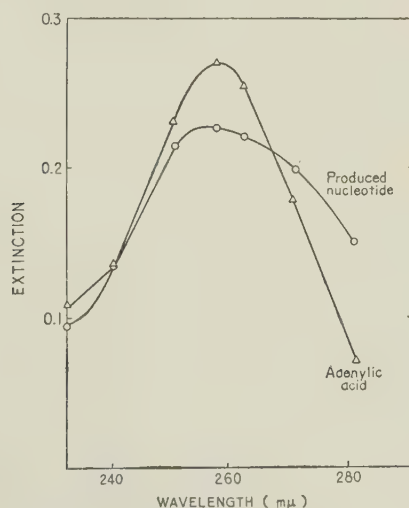


FIG. 5. Absorption spectrum of the digestion product of yeast RNA by RNase T₂ comparing with that of adenylic acid. Solvent was 0.01 N HCl. —△—, adenylic acid; —○—, produced nucleotide.

hours at 37° and the mixture was neutralized. By these treatments mononucleotides and terminal nucleotides were converted to nucleosides and internal nucleotides to mononucleotides. The nucleosides were detected by paper chromatography. A large amount of adenosine was produced from early stage, on the other hand other nucleosides appeared very slowly (Fig. 6). Each spot was cut off, eluted and quantity of each nucleoside was estimated spectrophotometrically.

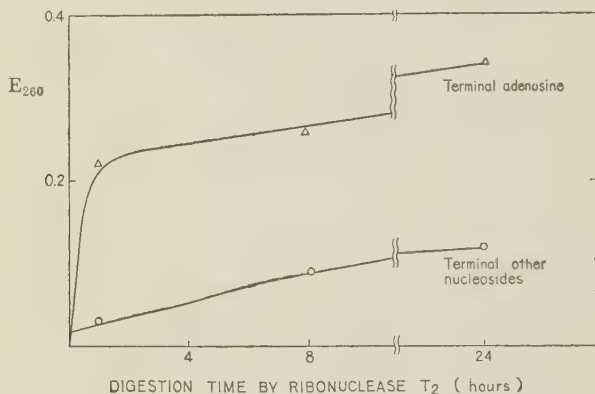


FIG. 6. Terminal nucleosides produced by the splitting of terminal phosphate by PMase and alkaline hydrolysis from oligonucleotides produced by RNase T₂. Initial reaction mixture is the same as in Fig. 4. PMase treatment was carried out for 2 hours with about 40 units of enzyme (8). Aliquots of the final reaction mixture were chromatographed using the solvent; *n*-butanol:water:ammonium hydroxide (86:14:5) (9), and each spots was eluted by 0.01 *N* HCl for 48 hours. The optical density at 260 mμ was measured. —△—, adenosine; —○—, other nucleosides.

OTHER OBSERVATIONS AND DISCUSSION

As described in the previous reports RNase T₁ hydrolyses specifically the secondary phosphate ester bond of 3'-guanylic acid and produces rapidly 3'-guanylic acid from yeast RNA *via* guanosine-2', 3'-cyclic phosphate (2, 3). On the other hand, RNase T₂ hardly produced mononucleotides from yeast RNA, and all oligonucleotides produced by RNase T₂ digestion were found to have adenylic acid as the terminal residue. So it is concluded that RNase T₂ preferentially hydrolyses the phosphodiester bond of adenosine-3'-phosphate among the internucleotides linkages and it hydrolyses very slowly, if any, that of the other nucleotides.

It has been not yet finally established whether RNase T₂ produces nucleoside-2', 3'-cyclic phosphate during the digestion of RNA or not. RNase T₂ hydrolysed adenosine-2' 3'-cyclic phosphate synthesized by the method of Brown *et al.* (10) to 3'-adenylic acid and it catalysed the synthetic reaction producing oligonucleotides from the cyclic phosphate (11). The formation

of a cyclic intermediate could not be detected so far by paper chromatography during the course of digestion of yeast RNA. Besides this, two procedures were applied to verify the existence of cyclic intermediate (i) Yeast RNA was digested by RNase T₂ for various hours as described above and terminal phosphomonoesters were examined by PMase method (3). After HCl treatment inorganic phosphate liberated by PMase did not increase distinctly during the course of digestion by RNase T₂. So cyclic nucleotides as the terminal of the oligonucleotides could not be identified so far under the condition described above. (ii) Polyadenylic acid (kindly supplied by Prof. S. Ochoa) synthesized by polynucleotide phosphorylase (12) was subjected to the action of RNase T₂ for 1 and 20 hours. In the hydrolysate only adenylic acid, but not oligoadenylic acid, was found by paper chromatography using solvent; isopropanol:water, 7:3; ammonia with vapour phase (13). From these results it seems that either RNase T₂ does not produce cyclic phosphate as an intermediate, or it produces cyclic phosphate splitted too rapidly to be observed.

When RNA was digested by the crude RNase T₂ fraction, about 90 per cent phosphate of total phosphate became uranium reagent soluble. By chromatographic studies adenylic acid appeared at first, increased gradually and pyrimidine nucleotides followed it. And after 24 hours digestion four mononucleotides appeared. On the contrary pyrimidine nucleotides did not appear in the digestion of yeast RNA by pure RNase T₁, pure RNase T₂ and their mixture. Therefore another RNase, which hydrolyses the phosphodiester bonds of pyrimidine nucleotides, may exist. It is very interesting that there are so many different RNases in the same source.

These RNases may be very useful for the study of the structure of polynucleotides. For instance a large amounts of guanylic acid was produced as mononucleotide by RNase T₁ digestion of yeast RNA, while adenylic acid was scarcely liberated as mononucleotide by RNase T₂ digestion, suggesting the different mode of distribution of guanylic acid and adenylic acid in yeast RNA.

SUMMARY

1. RNase T₂ was partially purified from Takadiastase. Most purified preparation was almost homogeneous in zone-electrophoresis and free from RNase T₁.

2. RNase T₂ is heat stable and has pH optimum at 4.5. It is inhibited by Cu⁺⁺ and activated by EDTA, PCMB and monoiodoacetate.

3. Specificity of RNase T₂ is very characteristic, namely it preferentially hydrolyses secondary phosphate ester bonds of a'-enosine-3'-phosphate.

4. RNase T₂ digestion of yeast RNA is not complete and a resistant fraction remains.

5. In the crude RNase T₂ fraction the existence of small amount of another RNase, perhaps hydrolysing phosphodiester bonds of pyrimidine

nucleotides, was suggested.

The authors wish to thank Sankyo Co., Ltd. for the gift of "Takadiastase Sankyo".

REFERENCES

- (1) Sato, K., and Egami, F., *J. Biochem.*, **44**, 753 (1957)
- (2) Sato, K., and Egami, F., *Compt. rend. soc. biol.*, **151**, 1792 (1957)
- (3) Sato-Asano, K., *J. Biochem.*, **46**, 31 (1959)
- (4) Sato-Asano, K., and Egami, F., *Biochim. et Biophys. Acta*, **29**, 655 (1958)
- (5) Sarkar, N. K., and Sumner, J. B., *Enzymologia*, **14**, 280 (1951)
- (6) Peterson, E. A., and Sober, H. A., *J. Am. Chem. Soc.*, **78**, 751 (1956)
- (7) Markham, R., and Smith, J. D., *Biochem. J.*, **49**, 401 (1951)
- (8) Smidt, G., Cubiles, R., Zöllner, N., Hecht, L., Strickler, N., Seraidarian, K., Seraidarian, M., and Thanhauser, S. J., *J. Biol. Chem.*, **192**, 715 (1951)
- (9) Markham, R., and Smith, J., *Biochem. J.*, **45**, 294 (1949)
- (10) Brown, D. M., Magrath, D. I., and Todd, A. R., *J. Chem. Soc.*, 2708 (1952)
- (11) Naoi, M., Asano, K., and Egami, F., Seventh Symposium on Nucleic Acid, of Japanese Association for Nucleic Acid Research, February, 1958
- (12) Grunberg-Manago, M., Ortiz, P. J., and Ochoa, S., *Science*, **122**, 907 (1955); *Biochim. et Biophys. Acta*, **20**, 269 (1956)
- (13) Markham, R., and Smith, J. D., *Biochem. J.*, **52**, 558 (1952)

2-AMINOETHANESULFINIC ACID

I. ISOLATION FROM A MOLLUSC; IDENTIFICATION AND DISTRIBUTION

By SHUNJI OUCHI

(From the Department, Faculty of Science, Kyushu University, Fukuoka)

(Received for publication, October 20, 1958)

In 1951, Bergeret and Chatagner (1) observed by paper chromatographic analysis that 2-aminoethanesulfinic acid was formed from cysteinesulfinic acid by the action of acetone-dried liver preparation. They named the compound "hypotaurine", which is readily oxidized to taurine. Furthermore, in 1953 Awapara (2) isolated this compound from rat liver after injection of cysteine, and in 1955 Cavallini *et al.* (3) isolated the same compound from urine of rats fed with cystine. 2-Aminoethanesulfinic acid, however, has never been reported to be isolated directly from organisms under natural conditions without administration of the precursors such as cysteinesulfinic acid, cysteine or cystine.

The present author, in the course of his studies on sulfur-containing compounds and their metabolism, found that paper chromatographic analysis of extracts from *Septifer* (*Mytilisepta*) *virgatus* (Wiegmann) by means of the iodine-azide reaction (4) showed the presence of an unusual sulfur compound, and that every tissue of this *Septifer* contained this compound in considerable quantities. The sulfur-containing compound was isolated and identified as 2-aminoethanesulfinic acid.

In the present paper, the isolation, identification and distribution of 2-aminoethanesulfinic acid are presented.

EXPERIMENTALS

Isolation—Shells of the *Septifer* were removed and the contents were ground in a mixer with water. The extract was deproteinized with ethanol and centrifuged. The precipitate separated from the supernatant liquid was washed with water, and the washings, after deproteinization and centrifugation, were mixed with the supernatant liquid obtained above. To extract the substance completely, the same operations were repeated three to four times, and then the combined solution was passed through a column of Amberlite IR-120 in the acid form. After washing the column with water, the fraction containing the required compound was eluted with 1 *N* ammonia. The eluate was evaporated up to dryness under reduced pressure in an inert gas (which was used to prevent 2-aminoethanesulfinic acid from

being oxidized to taurine). The residue was then dissolved in a small amount of water, and after removal of the insoluble part, the solution was passed through a column of Amberlite IRC-50 (H-form). The resultant solution was again passed through a column of Amberlite IR-4B previously treated with a sodium hydroxide solution and washed with CO₂-free water. The latter two operations were repeated two to three times. By these procedures a considerable amount of impurity was removed. The fraction thus obtained was treated once more with a quantity of acid form of Amberlite IR-120, about ten times as much as necessary to absorb the compound completely. When eluted with about 0.01 *N* hydrochloric acid, the compound was found only in the first effluent portion. By passing this portion through a column of Amberlite IR-4B, the hydrochloric acid used as the eluting agent and a trace of taurine formed during the fractionation were removed. When the solution thus purified was concentrated to syrup under reduced pressure and then allowed to stand under anacrobic condition in an ice-box with addition of ethanol, there appeared colorless needle-like crystals of the sulfur-containing compound. Approximately 50 mg. was obtained from 1 kg. of fresh material without shells (paper chromatographic analysis indicated that the organism contained about 150 mg. of the substance per kg.). The isolate gave paper chromatographically only one spot with the following reagents: ninhydrine, iodine-azide (4), sodium nitroprusside-potassium ferricyanide*, iodoplatinate (5), KI+HCl, and FeCl₃ (3).

Identification—According to Crumpler and Dent's method (6), it was indicated that the isolate could not be an α -amino acid. When heated with

TABLE I
R_f Values of the Isolated and Synthetic 2-Aminoethanesulfinic Acid in Various Solvents (Paper: Toyo Roshi No. 3)

Solvent	<i>R_f Values</i>	
	Isolated	Synthetic
<i>tert.</i> -Butanol-formic acid-water (75:15:10)	0.34	0.34
<i>n</i> -Butanol-acetic acid-pyridine-water (30:6:20:24)	0.27	0.27
<i>n</i> -Butanol-acetic acid-water (4:1:1)	0.21	0.21
Lutidine-water (75:25)	0.19	0.19
<i>n</i> -Butanol-pyridine-water (1:1:1)	0.28	0.29
Phenol-water (4:1)	0.62	0.62

hydrogen peroxide, this compound was transformed easily and quantitatively into taurine. In view of the result of elementary analysis, the compound was concluded to be 2-aminoethanesulfinic acid, though some compounds

* In general this reagent is used for the coloration of guanidino compound.

such as mercaptoethylamine, cystamine and cystamine disulfoxide can also give rise to taurine on oxidation. The isolated 2-aminoethanesulfinic acid showed the same paper chromatographic behavior as the synthetic compound according to A w a p a r a 's method (2). The R_f values of the natural and the synthetic compounds in various solvent agreed satisfactorily with each other (Table I). Both the compounds gave also the same migration pattern on paper electrophoresis. The isolate melted at 175-177°, the value being in accordance with those reported by different authors (3, 7, 8).

Analysis Calcd. for $H_2NCH_2CH_2SO_2H$: C, 22.02; H, 6.42; N, 12.84; S, 29.36
 Found : C, 21.86; H, 6.48; N, 12.38; S, 29.84

Distribution in Marine organisms—Fresh tissues of various marine organisms were extracted with water and the extracts were purified by means of Amberlite IR-120, Amberlite IRC-50 and Amberlite IR-4B as described above. After being purified to a considerable degree, each extract was dissolved in water at the rate of 5 ml. of water per 25 g. of fresh material. 0.01 ml. of the solution was paper chromatographed in *tert.*-butanol/formic acid/water

TABLE II
*The Distribution of 2-Aminoethanesulfinic Acid
 in Marine-livings*

Phylum	Species	Distribution of 2-aminoethanesulfinic acid
Mollusca	<i>Septifer virgatus</i>	++
	<i>Mytilus edulis</i>	+
	<i>Trichomya hirusta</i>	+++
	<i>Barbatia obtusoides</i>	+
	<i>Adula strata</i>	+
	<i>Brachidontes senhousia</i>	—
	<i>Tegula argyrostoma bsilirata</i>	—
	<i>Monodonta labio</i>	—
	<i>Patelloida saccharina lanx</i>	—
	<i>Cellana nigrolineata</i>	—
	<i>Nerita japonica</i>	—
	<i>Thais clavigera</i>	—
	<i>Lunella coronata coreensis</i>	—
Coelenterata	<i>Anthopleura stella</i>	++
	<i>Diadumene luciae</i>	+
	<i>Heliocidaris crassispina</i>	—
	<i>Temnopleurus toreumaticus</i>	—
Annelida	<i>Loimia medusa</i>	—

(75:15:10) and *n*-butanol/acetic acid/pyridine/water (4:1:1:2). 2-Aminoethanesulfinic acid was detected by the use of both iodine-azide and sodium

nitroprusside-potassium ferricyanide reagents, giving the same R_f value as the synthetic compound.

Most of the molluscs living on rock which have byssus contained 2-aminoethanesulfinic acid but the other molluscs did not. Many of the sea-anemones (coelenterates) contained a considerable amount of the compound. The compound was also found in appreciable amounts in some unidentified species of sponges (poriferes). The echinodermites, *Heliocidaris crassispina* and *Temnopleurus toreumaticus* did not seem to contain the substance, but curiously enough, large amounts of this compound was found in some preparations of salted sea-urchin purchased in local markets. The distribution of the substance in the marine organisms are summerized in Table II.

DISCUSSION

The presence of 2-aminoethanesulfinic acid in *Septifer* is decisive from the data presented above. Since the organism containing 2-aminoethanesulfinic acid had also taurine, the occurrence of the former compound in the organism suggests that it may play a role in the metabolism leading from cysteine or cystine to taurine. It is of interest that an easily oxidizable compound such as 2-aminoethanesulfinic acid exists in some marine-organisms in relatively high content, though in other animals the compound is rapidly oxidized and excreted (9, 10, 11). However, it is not yet clear whether or not the 2-aminoethanesulfinic acid contained in these organisms has been derived from cysteine or cystine, and whether or not it is oxidized only to taurine. Further work is necessary to elucidate these points.

Only one report has been presented by Robin and Roche (12) in respect to 2-aminoethanesulfinic acid in marine-organisms. Within the limits of the present investigation, there could not be found so many organisms containing appreciable amounts of this compound. However, it is considered that the organisms which contain the substance in an amount not detectable by the method described above may exist in a large number.

SUMMARY

2-Aminoethanesulfinic acid was isolated in pure form from a mollusc. The isolated compound was identical with synthetc 2-aminoethanesulfinic acid, as shown by comparison on the paper chromatography, paper electrophoresis and other properties.

The distribution of 2-aminoethanesulfinic acid in some marine-livings was also investigated.

The author wishes to express here his thanks to Prof. S. Shibuya for his suggestions and advices in the course of this work. The cost of this work has been partially covered by the Scientific Research Grant of Ministry of Education.

REFERENCES

- (1) Bergeret, B., and Chatagner, F., *C. R. Acad. Sci., (Paris)*, **232**, 448 (1951); *Biochim. et Biophys. Acta*, **9**, 141, 147 (1952)
- (2) Awapara, J., *J. Biol. Chem.*, **203**, 183 (1953)
- (3) Cavallini, D., Mondovi, B., and De Marco, C., *J. Biol. Chem.*, **216**, 577 (1955)
- (4) Feigl, F., *Spot Tests, 2. Organic Applications*, translated by R. E. Oesper, Elsevier Pub. Co., Amsterdam, Houston, London, New York, 164 (1954)
- (5) Winegart, H. M., Toennis, G., and Block, R. J., *Science*, **108**, 506 (1948)
- (6) Crumpler, H. R., and Dent, C. E., *Nature*, **164**, 441 (1949)
- (7) Eldjarn, L., and Sverdrup, A., *Acta Chem., Scand.*, **9**, 1037 (1955)
- (8) Bricas, E., Kieffer, F., and Fromageot, C., *Biochim. et Biophys. Acta*, **18**, 358 (1955)
- (9) Awapara, J., and Wingo, W. J., *J. Biol. Chem.*, **203**, 189 (1953)
- (10) Cavallini, D., De Marco, C., Mondovi, B., and Stirpe, F., *Biochim. et Biophys. Acta*, **15**, 301 (1954)
- (11) Eldjarn, L., Pihl, A., and Sverdrup, A., *J. Biol. Chem.*, **223**, 353 (1956)
- (12) Robin, Y., and Roche, J., *Compt. rend. soc. biol.*, **143**, 1783 (1954)

HEME SYNTHESIS IN THE SOLUBLE PREPARATION FROM AVIAN ERYTHROCYTES*

By YOSHIKO KAGAWA, SHIGEKI MINAKAMI AND
YOSHIMASA YONEYAMA

(From the Department of Physiological Chemistry and Nutrition,
Faculty of Medicine, University of Tokyo, Tokyo)

(Received for publication, October 21, 1958)

Although the outline of porphyrin biosynthesis has been elucidated by the extensive investigation of Shemin (1), Rimington (2) and other workers, the mechanism of hemoglobin-synthesis still remains to be studied. For example, the way that the three components of hemoglobin, *i.e.* porphyrin, iron and globin, combine to form hemoglobin, is not yet elucidated. The hypothesis, that the final process of hemoglobin synthesis be the insertion of iron into protoporphyrin-globin complex, was proposed by Eriksen (3), contrary to the general apprehension that iron and porphyrin combine at first to form heme and then it coordinates to globin. In addition whether protoporphyrin is the real precursor of heme or not, is still controversial. The solution of these problems may be partly attained by the investigation on the mechanism of the iron insertion into porphyrin ring, because the step of iron insertion may play an important role on the combination of protein and prosthetic group.

Insoluble particles of the hemolysate of avian erythrocytes have been known to possess the ability to incorporate radioactive iron into heme or presumably into hemoglobin *in vitro*. The insoluble system should be converted into soluble form for the study of the iron inserting mechanism. The active substance which plays a role for the insertion of iron into porphyrin has been extracted with Tween 20 from the insoluble particles of duck hemolysate by Krueger, Melnick and Klein (4).

In the previous communication from the authors' laboratory, the investigation on the incorporation of radioactive iron into heme by canine reticulocytes and by the hemolysate of duck erythrocytes was reported (5, 6). The present investigation deals with (i) the preparation of the active principle from the particulate fraction of avian hemolysate, (ii) some properties of the preparation and finally (iii) the influence of several inhibitors and activators on the activity of the preparation.

MATERIALS AND METHODS

The preparation obtained from hemolysate was incubated at 37° after addition of

* A part of this study has been already published in Japanese (11, 12).

radioactive iron (Fe^{59}) and protoporphyrin. The percentage of the iron incorporation into heme was calculated from the total hemoglobin content of the incubation mixture and the specific activity of the crystalline hemin prepared from the mixture incubated. The amount newly synthesized heme may be calculated from the amount of iron added, provided that the iron present in the preparation was negligible. The details of the procedure are as follows:

Preparation of Hemolysate and Solubilization—Fresh blood was taken from jugular vein of normal adult chickens or ducks (chicken was used in the experiment unless otherwise noted) into a bottle containing 15 mg. of penicillin G, 15 mg. of streptomycin and 10 mg. of heparin for every 100 ml. of the blood. The cells were collected by centrifugation and washed with isotonic potassium chloride solution. The leucocytes were removed by suction. To one volume of red cells two volumes of cold glass-distilled water were added and stirred. After standing for 1 hour in a refrigerator, potassium chloride solution was added to the hemolysate to make the mixture isotonic.

The hemolysate was centrifuged at 3,000 r.p.m. for 10 minutes. The supernatant was used as a carrier hemoglobin. The precipitate was washed several times with cold isotonic potassium chloride solution equal in volume to the supernatant. This insoluble particles were suspended in 1/15 *M* phosphate buffer solution (pH 7.2) made isotonic with potassium chloride and containing 0.5 per cent of sodium cholate. The suspension was placed in a refrigerator overnight and the supernatant was collected by centrifugation at 3,000 r.p.m. for 30 minutes. The soluble preparation thus obtained is clear red and somewhat viscous. Enhancement of the activity of the preparation was observed by the addition of ascorbate to the cholate solution in the course of the extraction and will be referred to later. This procedure was used in the latter part of the investigation.

The hemoglobin content of the preparation was determined photometrically at 540 $m\mu$. using a Klett-Summerson electro-photometer by pyridine-hemochromogen method.

Incubation—Five to seven ml. of the preparation was incubated with 1 ml. of 1/15 *M* phosphate buffer (pH 7.2), 1 ml. of potassium chloride solution of substrate, and 1 ml. of radioactive iron (approximately 5,000–500,000 c.p.m.) in 30 ml. conical flasks for 3–4 hours, shaking at 37°.

Isolation, Crystallization and Determination of Specific Activity—After the incubation 3–5 ml. of hemoglobin solution was added to the incubation mixture and the protein was precipitated by addition of acetone. The precipitated hemoglobin was treated as described in the previous report (6) and the crystalline hemin was prepared. The determination of specific activity was made also by the method stated in the same report.

Substrate—Protoporphyrin was prepared as reported in the previous publication (6). Porphobilinogen was the generous gift of Dr. G. Urata, which was prepared from δ -aminolevulinic acid using rat liver homogenate. δ -Aminolevulinic acid was the product of Daiichi Pure Chemical Company, Ltd., Tokyo*.

RESULTS

Solubilization of the Active principle for Heme Synthesis—As the most of the activity to incorporate radioactive iron into heme was observed in the insoluble fraction of the avian hemolysate (Table I). The system responsible for this activity should be solubilized for the further investigation. Sodium cholate, Tween 20, Tween 40 and taurocholate could be used as detergents

* The authors acknowledge the gift of δ -aminolevulinic acid to Daiichi Pure Chemical Company, Ltd., Tokyo.

for the solubilization. The reproducible results were obtained by the treatment with sodium cholate at pH 7.5 overnight in a refrigerator (Table II) and this procedure was used throughout this investigation. The several washing of the precipitate with isotonic potassium chloride solution could reduce the hemoglobin content of the preparation to some extent without considerable loss of the iron incorporating activity.

TABLE I
Iron Incorporation Activity of Hemolysate

Preparation	Iron incorporated (%)
Hemolysate	4.2
Supernatant of hemolysate	0.2
Particulate of hemolysate	4.2

3.5 ml. of hemolysate, supernatant and precipitate obtained from 3.5 ml. of hemolysate were used. Protoporphyrin concentration: 1.4×10^{-5} M. Incubation period: 3 hours.

TABLE II
Extraction of an Active Factor of the Heme Formation with Several Detergent from Insoluble Particle of Hemolysate

Exp. No.	Detergent	pH	Iron incorporated (%)
1	Tween 20	7.0	34
	Taurocholate	7.0	11
14	Cholate	7.0	28
	Tween 20	7.0	13
	Tween 40	7.0	15
4	Cholate	6.0	4.6
		6.5	4.1
		7.0	5.7
		7.5	7.8
		8.0	3.8
		8.5	5.7
		7.0	3.3

The precipitate of chicken hemolysate was suspended in isotonic buffered KCl solution containing detergent. The final concentration of each detergent was 0.4 per cent. The suspension was placed in a refrigerator overnight and the supernatant was incubated with radioactive iron and protoporphyrin (Exp. 1, 20×10^{-6} M, Exp. 4, 8×10^{-6} M, Exp. 14, 14×10^{-6} M). Incubation period (Exp. 1, 5 hours Exp. 4 and Exp. 14, 4 hours).

Some Properties of the Soluble Preparation—The incorporation of radioactive iron into heme was observed by incubating the preparation with protopor-

phyrin and radioactive iron, shaking at 37°. The increase of the radioactivity of heme was almost linear for at least 4 hours as shown in Fig. 1. The optimum pH for the incorporation lies between 7.0 and 7.4 (Fig. 2) and this value fairly differs from that of Krueger *et al.* (4), who have studied on the Tween 20 extract of duck erythrocytes. No incorporation resulted from the incubation in a refrigerator overnight. The preparation was completely inactivated by heating for 1 minute in a boiling water bath. The active principle could be precipitated by salting out with ammonium sulfate: by the 50 per cent saturation about 60 per cent of the activity was precipitated.

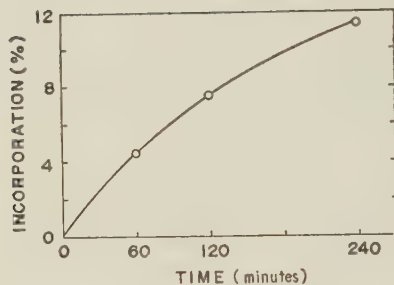


FIG. 1. Time course of the iron incorporation by the cholate extract (protoporphyrin concentration $8.3 \times 10^{-6} M$).

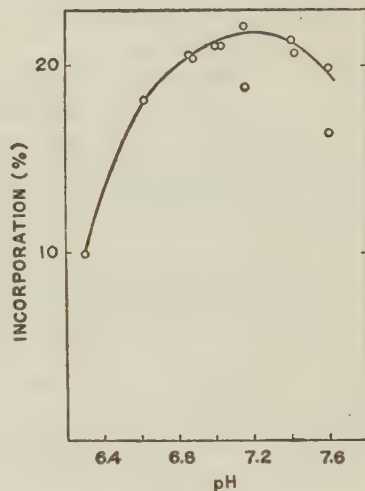


FIG. 2. The effect of pH on the iron incorporation (protoporphyrin concentration $1.4 \times 10^{-5} M$).

Five to ten per cent of the activity was lost per day when the preparation was kept in a refrigerator. By lyophilization of the preparation, only 10 per cent of the activity remained. More than half of the activity was lost by dialysis at 0–4° against glass-distilled water for one hour. The addition of the concentrated solution of the dialysable portion or cysteine did not cause any reactivation. However, the loss of the activity was somewhat prevented by the use of buffered isotonic potassium chloride solution (pH 7.2) containing 1 per cent of sodium cholate, instead of glass-distilled water.

The Effect of the Protoporphyrin and Iron Concentration—No incorporation of radioactive iron was observed by the incubation of the cholate extract without addition of protoporphyrin (Table III) and about $5 \times 10^{-5} M$ concentration of protoporphyrin was necessary for maximum incorporation of radioactive iron, as indicated by Fig. 3. The intermediates of porphyrin biosynthesis such as δ -aminolevulinic acid and porphobilinogen were also utilized in this system as a substrate for heme synthesis (Table III).

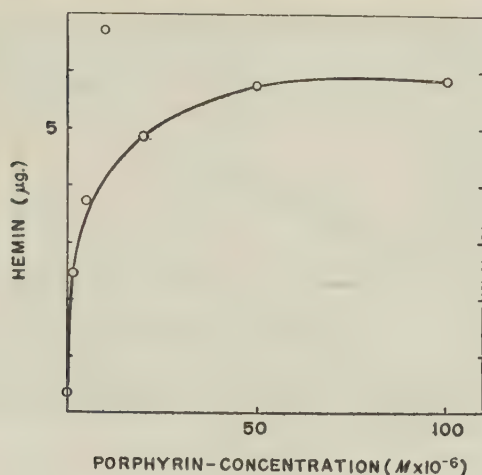


FIG. 3. The effect of porphyrin concentrations on the hemin synthesis. The preparation extracted from duck hemolysate was used for this experiment. 1 $\mu g.$ of iron as ferric ammonium citrate was added as carrier.

TABLE III

Effect of Several Substrates on Heme Synthesis

Exp. No.	Substrate	Concentration (M)	Iron incorporated (%)
5	Protoporphyrin	8.3×10^{-6}	8.4
	No substrate	0	0
6	Protoporphyrin	8.3×10^{-6}	6.0
	Porphobilinogen	17×10^{-6}	5.0
	δ -Aminolevulinic acid	66×10^{-6}	10.5

Incubation period: 4 hours.

The increase of the iron concentration has also caused the increase of the incorporation of the radioactive iron into heme as shown in the double reciprocal plot (Fig. 4). (The erythrocytes of duck, instead of chicken, were used in the experiment of Figs. 3 and 4).

The Effect of Reducing Agents—Reducing agents and anaerobic condition were found to be favorable for the heme synthesis in this system. The addition of ascorbate to the cholate solution caused a marked increase of the activity of the extract (Table IV). From this finding this procedure was used in the latter part of the investigation (Exp. 19 and afterwards). The addition of ascorbate during the incubation, did not accelerate the reaction.

A remarkable accelerating effect of cysteine was observed when the preparation was not pretreated with ascorbate. Only a negligible acceleration

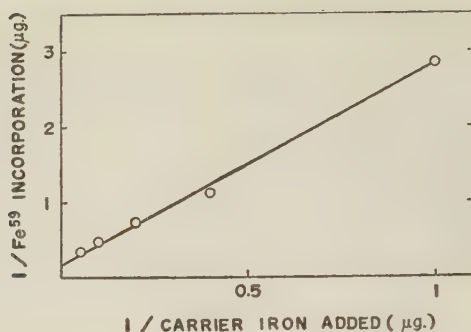


FIG. 4. Double reciprocal plot showing the effect of iron concentrations on the iron incorporation. The preparation extracted from duck hemolysate was used for this experiment (final concentration of protoporphyrin: $1 \times 10^{-5} M$).

TABLE IV
Effect of Ascorbic Acid Treatment

Preparation	Iron incorporated (%)
Without the treatment	17.4
$10^{-3} M$ ascorbic acid was added to the cholate solution before extraction	30.4

Concentration of protoporphyrin: $1.4 \times 10^{-5} M$.
Incubation period: 3 hours.

TABLE V
Effect of Nitrogen and Reducing Agents on Heme Formation

Exp. No.	Gas-phase	Addition	Iron incorporated (%)
7	Air		5.6
	Air	Ascorbic acid $10^{-2} M$	8.8
	Air	Cystein $10^{-2} M$	25.0
10	Air		11.3
	Nitrogen		14.7
19*	Air		34.0
	Nitrogen		37.0
	Air	Cystein $10^{-3} M$	40.2
	Nitrogen	Cystein $10^{-3} M$	37.3
	Air	Glutathione $10^{-3} M$	39.5
	Nitrogen	Glutathione $10^{-3} M$	39.2

Protoporphyrin concentration: Exp. 7, $8.3 \times 10^{-6} M$, Exp. 10, $5.5 \times 10^{-6} M$, Exp. 19, $14 \times 10^{-6} M$, Incubation period: Exp. 7 and Exp. 10, 4 hours, Exp. 19, 3 hours.

* Ascorbic acid treated preparation was used.

was observed when the ascorbate-pretreated preparation was used. The effect of glutathione was similar to that of cysteine (Table V).

Effects of Some Inhibitors—A summary of the effects of various inhibitors on the incorporation of iron into heme is given in Table VI.

TABLE VI
Effects of Inhibitors

Exp. No.	Inhibitor	Molarity	Percent activity
	Control		100
6	Lead in Tris buffer	1×10^{-2}	9
		1×10^{-3}	13
		1×10^{-4}	32
		1×10^{-5}	68
6	Lead in phosphate buffer	1×10^{-2}	12.5
		1×10^{-3}	37.5
		1×10^{-4}	55
		1×10^{-5}	63
9	Copper	1.5×10^{-5}	86
10	Copper	1.5×10^{-4}	35
20	Mercury	1×10^{-3}	36
		1×10^{-4}	70
		1×10^{-5}	82
15	EDTA	1×10^{-2}	0
19	Monoiodoacetate	1×10^{-2}	76
		1×10^{-3}	91
		1×10^{-4}	99
12	PCMB	3×10^{-3}	12
		1×10^{-3}	42
		1×10^{-4}	87
19	Cyanide	1×10^{-2}	108
12	Cyanide	1×10^{-3}	106
		1×10^{-4}	96
9	Dinitrophenol	1×10^{-2}	85
	Malonate	1×10^{-2}	74
14	Chicken plasma	10% (v/v)	0

The most prominent inhibitor was found to be lead acetate, which inhibited the reaction about 68 per cent at $10^{-5} M$ concentration if Tris (tris-hydroxymethylaminomethane) buffer was used. The lesser inhibition was observed when phosphate buffer was used instead of Tris buffer; the

former is known to precipitate lead ion. Other heavy metals, such as copper or silver ion also inhibited the reaction, though they were not so effective as lead. EDTA (ethylenediaminetetraacetic acid) completely inhibited the incorporation at $10^{-2} M$ concentration. This inhibition may probably be due to the chelation of EDTA to the radioactive iron. The most unexpected result was that chicken plasma exerted powerful inhibitory effect, which may be also due to the chelating effect of the protein.

Among the inhibitors known to react with sulfhydryl groups *p*-chloromercuribenzoate was effective, while monoiodoacetate was almost ineffective as an inhibitor. Cyanide has been known to be a potent inhibitor for heme synthesis in hemolysate or whole blood cells (5, 6), whereas in this system cyanide showed no inhibitory effect and even a slight acceleration was observed in some cases. 2,4-Dinitrophenol and malonate exerted only slight inhibition even at high concentrations.

DISCUSSION

In the present investigation a soluble preparation having a synthetic activity of heme from iron and protoporphyrin was extracted from the particulate fraction of avian hemolysate. This preparation was heat labile, had an optimum pH for the activity and could be precipitated by the addition of ammonium sulfate. The possibility that the active substance be a protein may be suggested from the above findings. However, the protein is not necessarily an enzyme because it is not yet certain whether this substance may be used as a raw material or as a catalyst for hemoglobin synthesis. Further experiments, especially the purification, will be necessary to elucidate the nature of the active principle.

It is still controversial whether the protoporphyrin is a direct precursor in heme synthesis or not. Dresel and Falk (7) expressed their opinion that protoporphyrin is not an intermediate in heme synthesis but merely a secondary product, from the observation that the radioactivity of heme synthesized from C^{14} -glycine, was not diluted by the addition of non-radioactive protoporphyrin. The formation of heme from reduced uroporphyrin was reported by Neve *et al.* (8), Neilands *et al.* (9) also observed the incorporation of iron into the precursor of protoporphyrin, which is not decarboxylated and presumably in a reduced state. As shown in the present experiment protoporphyrin was utilized for the synthesis of heme and according to the preliminary experiment of the authors, the radioactivity was introduced into heme from C^{14} -protoporphyrin.

As described above, reducing agents showed accelerating effect for the reaction. If the activation is caused by the reduction of porphyrin or ferric ion the ineffectiveness of ascorbate is obscure. The activation effect of cysteine was observed only when the preparation was not pretreated with ascorbate and the activity was low. It is considered that the active substance has some easily oxidizable part and the reduced state is necessary for the

reaction. It is possible that anaerobic condition and ascorbate only protect the oxidation, but cysteine reactivate the oxidized preparation. The participation of sulfhydryl group in the reaction may be suggested. Inhibition by *p*-mercuribenzoate and heavy metal supports this suggestion.

Dresel and Falk (10) reported that the heme synthesis by avian hemolysate was inhibited by lead at the step of ALA-dehydrase. In this experiment, lead inhibited specifically the insertion of iron into heme. Complexity of the pathogenesis of the anemia due to the lead poisoning may be concluded. The incorporation was completely inhibited by the addition of blood plasma, contrary to the findings of several investigators that blood plasma accelerate hemoglobin synthesis *in vitro*. Presumably some protein in the plasma combined with iron to inhibit the reaction.

At this stage of heme synthesis, that is the insertion of iron into protoporphyrin, it is considered that the reaction does not require energy. Therefore dinitrophenol and malonate was expected to exert no inhibiting effect. Dinitrophenol and malonate showed slight inhibitory effects at high concentrations. This slight inhibition may not be due to the effect on energy yielding system but may depend on another mechanism.

SUMMARY

1. An active preparation to synthesize heme from protoporphyrin and iron was solubilized from particulates of avian hemolysate using sodium cholate solution.

2. The preparation has an optimum pH for the activity and is completely inactivated by heat treatment.

3. Protoporphyrin was required as a substrate, though δ -aminolevulinic acid or porphobilinogen could replace it in the present system.

4. The reaction was remarkably accelerated by addition of cysteine or glutathione. The addition of ascorbate in the course of the cholate extraction showed enhancement of the activity of the preparation.

5. Lead, silver, copper, ethylene diamine tetraacetic acid, *p*-cholomer-curibenzoate and chicken serum considerably inhibited the reaction, while 2,4-dinitrophenol, malonate and cyanide showed slight or no inhibitory effects.

The authors express their thanks to Prof. H. Yoshikawa for his constant advice and encouragement during the course of the work. Thanks are also due to Dr. G. Ura ta of the Institute of Public Health for his valuable discussions and also for the generous gift of porphobilinogen and to Mr. S. Tanaka, Dr. Y. Sugita and Dr. S. Morimoto of this laboratory for their stimulating discussions.

REFERENCES

- (1) Shemin, D., in "Porphyrin Biosynthesis and Metabolism" Ciba foundation symposium, J. & A. Churchill, London p. 4 (1955)
- (2) Rimington, C., International Symposium on Enzyme chemistry Tokyo, October, (1957)

- (3) Eriksen, L., in "Porphyrin Biosynthesis and Metabolism", Ciba foundation symposium, J. & A. Churchill, London, p. 186 (1955)
- (4) Krueger, R. C., Melnick, J., and Klein, J. R., *Arch. Biochem. Biophys.*, **64**, 302 (1956)
- (5) Yoshida, A., Hattori, M., Nokao, K., Kagawa, Y., Morimoto, S., Minakami, S., and Yoneyama, Y., *J. Japan. Biochem. Soc.*, **30**, 197 (1959)
- (6) Minakami, S., Kagawa, Y., Tanaka, S., Sugita, Y., and Yoneyama, Y., *J. Biochem.*, **45**, 899 (1958)
- (7) Dresel, E. I. B., and Falk, J. E., *Biochem. J.*, **63**, 388 (1956)
- (8) Neve, R. A., Labbe, R. F., and Aldrich, R. A., *J. Am. Chem. Soc.*, **78**, 691 (1956)
- (9) Neilands, J. B., International Symposium on Enzyme Chemistry, Tokyo, October, (1957)
- (10) Dresel, E. I. B., and Falk, J. E., *Biochem. J.*, **63**, 80 (1956)
- (11) Kagawa, Y., Minakami, S., Urata, G., Tanaka, S., Sugita, Y., Morimoto, S., and Yoneyama, Y., *J. Japan. Biochem. Soc.*, **30**, 292 (1958)
- (12) Kagawa, Y., *J. Japan. Biochem. Soc.*, **30**, 332 (1958)

After the manuscript had been prepared, the papers of Heikel and others (*Nature*, **182** 313 (1958)) became accessible to the authors, in which the non-enzymic formation of heme in the presence of surface-active agent was reported. The incorporation of radioactive iron into heme observed by the authors, however, was not due to the presence of a surface-active agent, sodium cholate. The experiment with radioactive ferric chloride in the presence of sodium cholate and cysteine did not show any detectable incorporation into heme.

STUDIES ON ENZYMIC NITRITE REDUCTION

I. PROPERTIES OF THE ENZYME SYSTEM INVOLVED IN THE PROCESS OF NITRITE REDUCTION*

By AKIRA ASANO

(From the Department of Chemistry, Faculty of Science,
Nagoya University, Nagoya)

(Received for publication, October 27, 1958)

While the physiological meaning of nitrate reduction and its enzymic mechanism have recently been clarified considerably (1-3), the process of nitrite reduction have drawn relatively less attention (4-9). Nitrite is important in nitrate reduction process, being a key intermediate lying on the pathway from nitrate to either hydroxylamine and ammonia or gaseous products such as nitrogen, nitrous oxide *etc.* In the case of denitrification nitrite reduction may be an energy-yielding process like aerobic respiration, so it is interesting to study the electron transport system functioning in the reduction of nitrite in aerobic denitrifiers comparing it with that of aerobic respiration. In this report, the participation of pyridine nucleotide, flavin, vitamin K, cytochrome and some heavy metal in the electron transport system will be described.

MATERIALS AND METHODS

Micro-organism—A halotolerant *Micrococcus* strain 203 was cultured aerobically, harvested, and lysed by dialysing the suspension of lyophilized cells as described in the communication by Kono *et al.* (10).

Enzyme Preparation—Dialysed cells were centrifuged at $20,000 \times g$ for 30 minutes at 0° . Electron transport system and nitrite reductase activity were partially extracted by this procedure. To the supernatant (crude extract) phosphate buffer pH 6.8 and sodium chloride were added to the final concentration of 0.02 *M* and 0.3 *M*, respectively, and an equal volume of cold acetone was added. The precipitate formed was separated by centrifugation and dissolved in 0.02 *M* phosphate buffer (pH 6.8). By this procedure the specific activity measured by MbH_2 method increased about 2-fold compared to that caused by lyophilized cells and recovery of the activity was 30 per cent. When nitrite reductase activity had not been extracted effectively by this method, the dialysed cells were centrifuged at $5,000 \times g$ for 30 minutes. The precipitate was suspended in water and exposed to ultrasonic oscillation (490 kc) for 30 minutes. Cell debris were centrifuged off at $20,000 \times g$ for 30 minutes. To the supernatant, phosphate buffer and sodium chloride were added to the final concentration of 0.01 *M* and 0.05 *M*, respectively, and the precipitate obtained between 50 and 70 volume percent cold acetone was collected, and dissolved in 0.02 *M*

* A part of this work was reported on the 10th Symposium on Enzyme Chemistry held at Sapporo in July, 1958.

phosphate (pH 6.8). By this procedure the specific activity measured by the MbH_2 method increased about 7-fold compared with that of lyophilysed cells, and the recovery of the activity was 60 per cent.

Assay of Nitrite Reductase—Usual *Thunberg* technique was employed. Reaction mixture contained 2.5μ moles of sodium nitrite, 5.0μ moles of electron donor as mentioned below (or 25μ moles of glucose or succinate and 1.25μ moles of sodium chloride), phosphate buffer pH 6.8 (final concentration $0.05 M$) in the main tube and enzyme preparation in the side arm. Final volume was 2.5 ml. and reaction was carried out at 37° . Reaction was stopped by *Fujita-Iwatake's* cadmium sulfate solution neutralized to pH 4.2. Kaolin or active charcoal was used for decoloration. Nitrite was estimated according to *R. Novak* and *P.W. Wilson (11)*. Reduced forms of methylene blue, toluylene blue and pyocyanine were prepared by reduction with hydrogen in presence of palladium catalyst. 2,6-Dichlorophenol indophenol and phenazine methosulfate were reduced by ascorbic acid and methyl viologen was reduced with *Mg* powder. Phenazine methosulfate was synthesized from phenazine and dimethyl sulfate (12).

Denitrifying activity was measured by usual *Warburg* technique with nitrogen in the gas phase. Reaction mixture contained 5.0μ moles of sodium nitrite, 11.5μ moles of reduced methylene blue or 30μ moles of sodium formate, phosphate buffer pH 6.8 (final concentration $0.08 M$) in the main compartment and the enzyme preparation in the side arm. When sodium formate was used as an electron donor, sodium chloride was added to $0.5 M$ in final concentration. Total volume was 3.0 ml. and 0.1 ml. of 20 per cent *KOH* was placed in the center well. Reaction was carried out at 37° . Tocopherol suspension was prepared by the method described by *Nason et al. (15)*. Antimycin A and menadione were dissolved in absolute ethanol. The control experiments containing the same amount of ethanol were carried out.

RESULTS

Artificial Electron Donors—To study the nitrite reductase itself, it is necessary to assay the activity by simple and direct methods. For this purpose several reduced dyes and other electron donors were tested using the cell suspension. Among the two-electron donors, *p*-quinone ($E' = +0.690 \text{ v.}$ at pH 7), 2,6-dichlorophenol indophenol ($+0.271$), toluylene blue ($+0.115$), ascorbic acid ($+0.08$ at pH 6.43), methylene blue ($+0.011$), FAD^* (-0.06) and phenosafranine (-0.252), the most effective was reduced methylene blue; toluylene blue, FAD and phenosafranine were less effective, *p*-quinone, 2,6-dichlorophenol indophenol and ascorbic acid were ineffective. Among the one-electron donors, ferrocyanate ($+0.360$), phenazine methosulfate ($+0.080$), pyocyanine (-0.034) and method viologen (-0.446), only phenazine methosulfate was effective. Therefore, reduced methylene blue and reduced phenazine methosulfate were used as electron donors in the following experiments.

Product of Nitrite Reduction—Both cell suspension and purified enzyme preparation catalysed the production of one mole of nitrogen from two

* Following abbreviations were used; *NiR*, nitrite reductase; *FAD*, flavin adenine dinucleotide; *DPNH*, reduced diphosphopyridine nucleotide; MbH_2 , reduced methylene blue; *PMSH* reduced phenazine methosulfate; *Mb*, methylene blue; *PMS*, phenazine methosulfate; *cyt*, cytochrome; *HdR*, hydroxylamine reductase.

moles of nitrite (Fig. 1).

pH Optimum and Michaelis Constant—When MbH_2 was used as an electron donor, pH optimum was about pH 6.8 with phosphate buffer. Using the partially purified enzyme preparation, the Michaelis constant for nitrite and enzyme complex was found to be $5.6 \times 10^{-5} M$.

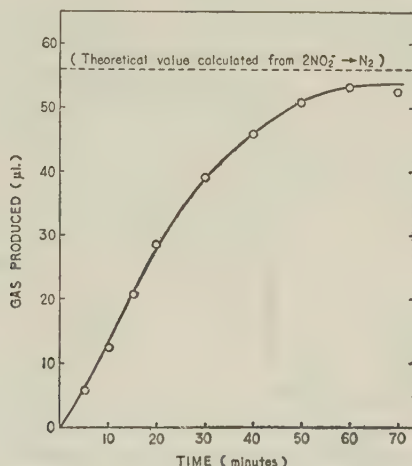


FIG. 1. Production of nitrogen from nitrite by nitrite reductase. Enzyme preparation used was a partially purified one which was prepared by acetone fractionation (12.5 mg. dry weight). Reaction was carried out under the condition as described in an earlier section.

Electron Transfer from Cytochrome b_4 to Nitrite—Crude extract contains a large amount of cytochrome b_4 * and very small amounts of other cytochrome(s). Cytochrome b_4 in the crude extract was reduced by ascorbic acid in an evacuated Thunberg-type cuvette and its reoxidation was observed by addition of nitrite. As shown in Fig. 2, when the reaction mixture contained a relatively large amount of sodium chloride, the reaction was very rapid, and at low sodium chloride concentration the reaction rate was greatly slowed down. Electron transfer from reduced cytochrome b_4 to nitrite could be observed using about 90 per cent pure cytochrome b_4 and purified NiR. NiR activity measured by the MbH_2 method, however, was not affected by sodium chloride concentration. Cytochrome b_4 oxidase was also shown to be halophilic by manometric method (13) and by spectrophotometric method.

Electron Transfer from DPNH via Cytochrome b_4 to Nitrite—Electron transfer from DPNH to nitrite did not occur in low sodium chloride concentration,

* Cytochrome b_4 has been divided into two different components. In this report cytochrom b_4 means cytochrome b_4 -I of R. Sato's. Its reduced form has absorption bands at 418 $m\mu$, 522 $m\mu$ and 544 $m\mu$ with a sub-peak at 548 $m\mu$.

but the electron transfer from DPNH to cytochrome b_4 was not affected by salt concentration. The following experiments concerning the electron transport system were carried out at 0.6 M of sodium chloride concentration.

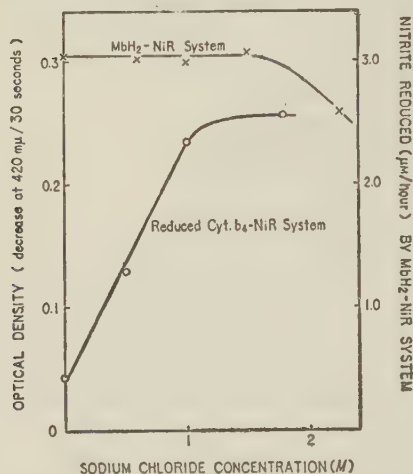


FIG. 2. Effect of sodium chloride concentration on cytochrome b_4 -NiR system and MbH_2 -NiR system. Reaction mixture of cytochrome b_4 -NiR system contained 1.0 μ moles of sodium nitrite, 1.5 μ moles of ascorbic acid, phosphate buffer pH 6.8 (final concentration 0.08 M), crude extract (1.5 ml.) and an adequate amount of sodium chloride. Total volume was 3.5 ml. Reaction was carried out in a Thunberg-type cuvette in anaerobic condition and at 15°C. Reaction mixture of MbH_2 -NiR system was as described in an earlier section with addition of adequate amounts of sodium chloride.

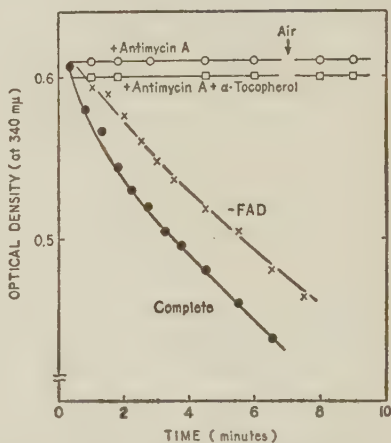


FIG. 3. Effect of FAD, antimycin A and α -tocopherol on DPNH-NiR system. Reaction mixture contained 1.0 μ moles of sodium nitrite, 0.3 μ moles of DPNH, 3.2 moles of sodium chloride, 5 μ moles of FAD, phosphate buffer pH 6.8 (0.08 M in final concentration) and 0.25 ml. of crude extract. Total volume was 3.2 ml. Reaction was carried out in a Thunberg-type cuvette under anaerobic condition and at 20°. 40 μ g. of antimycin A and 60 μ g. of α -tocopherol were added, if necessary.

Arrow indicates the introduction of air in the cuvette.

As shown in Fig. 3 the electron transfer from DPNH to nitrite and oxygen was completely inhibited by antimycin A (1 μ g./mg. dry weight) as in the case of mammalian tissue respiration (14). The inhibition could not be recovered by α -tocopherol. Addition of FAD at 1.7×10^{-3} M final concentration caused a two-fold activation of DPNH-nitrite reductase system at the initial stage. The rate of nitrite reduction of DPNH-nitrite reductase system was one third of that PMSH-NiR system.

Electron Transfer from Glucose to Nitrite—Using a dialysed cell suspension, effects of activators and inhibitors on the electron transport system were studied. As shown in Table I, amytal, quinine, dicumarol and antimycin A inhibited the system. Addition of FAD (4×10^{-4} M) and menadione (5×10^{-4} M) caused a marked stimulation. Methylene blue as an intermediary

electron carrier had the same effect. Inhibition by dicumarol ($2 \times 10^{-5} M$) (20) was completely overcome by the addition of menadione ($5 \times 10^{-4} M$). MbH_2 -NiR system and glucose-NiR system in the presence of methylene blue were not inhibited by antimycin A.

TABLE I
Effects of Inhibitors on Electron Transport System¹⁾

Electron donors	Intermediary electron carriers	Degree of activation (%)	Inhibitors	Concentration	Degree of inhibition (%)
Glucose			Antimycin A	1 $\mu g./mg.$	100
"			"	0.5 $\mu g./mg.$	90
"	Mb ($2 \times 10^{-4} M$)	150	"	—	—
"	"	—	"	1 $\mu g./mg.$	24
Succinate			"	1 $\mu g./mg.$	90
"			"	0.5 $\mu g./mg.$	60
"	PMSH ($4 \times 10^{-4} M$)	460	—	—	—
"	"	—	"	1.0 $\mu g./mg.$	7
MbH_2			"	0.5 $\mu g./mg.$	5
PMSH			"	1.0 $\mu g./mg.$	4
Glucose	—	—	Dicumarol	$10^{-4} M$	100
"	Menadione ($5 \times 10^{-4} M$)	—	"	"	100
"	—	—	"	$2 \times 10^{-5} M$	20
"	Menadione ($5 \times 10^{-4} M$)	280	—	—	—
"	"	—	"	$2 \times 10^{-5} M$	0
Succinate			"	$10^{-4} M$	0
Glucose	—	—	Amytal	$2 \times 10^{-3} M$	23
"	—	—	Quinine	$1 \times 10^{-3} M$	95
"	FAD ($4 \times 10^{-4} M$)	160	—	—	—

1) Enzyme preparation used was dialysed suspension of cell (50 mg/tube).

Electron Transfer from Succinate to Nitrite—Succinate could reduce cytochrome b in cell suspension and crude extract. Among the inhibitors, amytal, quinine, dicumarol and antimycin A, only antimycin A was effective. Addition of phenazine methosulfate markedly stimulated the activity and overcame the inhibition by antimycin A. PMSH-NiR system was not affected by antimycin A (Table I).

Effect of Inhibitors—As shown in Table II, metal binding agents such as cyanide and azide, and metal chelators inhibited the NiR activity in three different systems, MbH₂-NiR, PMSH-NiR and formate-Mb-NiR system measured by nitrogen gas production. Carbon monoxide in the dark inhibited at low concentrations and the inhibition was not removed by light. Nitric oxide inhibited the NiR at a very low concentration. Assuming that the solubility of nitric oxide in reaction mixture was the same as in water, its concentration under 0.1 atm. nitric oxide was 2×10^{-6} M at 37°. The results indicate that nitrite reductase has a heavy metal as an active center. Nitrous oxide did not affect the reaction rate.

SH-reagents did not shown significant inhibition. A great difference between MbH₂ system and PMSH system was observed in hydroxylamine

TABLE II
Effects of Inhibitors on NiR

Inhibitors	Concentration (M)	Degree of inhibition (%)		
		Electron donors		
		MbH ₂ ^{a)}	PMSH ^{b)}	Formate-Mb ^{c)}
KCN	10 ⁻²	100	100	65
	10 ⁻³	35	80	
NaN ₃	10 ⁻²	100		
	10 ⁻³	90	15	45
	10 ⁻⁴	15		
6-Phenanthroline	2 × 10 ⁻³	30	35	45
α,α'-Dipyridyl	2 × 10 ⁻³	20		35
	2 × 10 ⁻³		10	
Sodium diethyl dithiocarbamate	2 × 10 ⁻³	60		15
	10 ⁻³		15	
Salicyl aldoxime	10 ⁻³	25	30	
Allyl thiourea	10 ⁻³	30	0	
EDTA	5 × 10 ⁻³		0	
	2 × 10 ⁻³	30		
Dithizone	5 × 10 ⁻⁴	35		
PCMB	4 × 10 ⁻³	20	10	
NH ₂ OH	10 ⁻³	100	(5)	50
	10 ⁻⁴	100	(0)	
CO (light) ^{d)}	1 atm.	70	70	60
(dark)	"	70	70	60
(light) ^{d)}	1.0 atm.	30	35	
(dark)	"	40	35	
NO	1 atm.	—	100	
	0.1	100	45	

a), b) Enzyme preparation used was partially purified by acetone fraction.

c) Enzyme preparation used was cell suspension.

d) Tungsten lamp, 120 w. Distance, 20 cm.

inhibition. Hydroxylamine at 10^{-4} M inhibited the $\text{MbH}_2\text{-NiR}$ system completely, but PMSH-NiR system was not inhibited at all by hydroxylamine at 10^{-3} M. This result indicates that $\text{MbH}_2\text{-NiR}$ system may contain a hydroxylamine-sensitive intermediary electron carrier which is not required in PMSH-NiR system.

DISCUSSION

It appears evident from the preceeding results that the enzyme system transferring electrons to nitrite is closely related to mammalian terminal oxidase system. Participation of cytochrome(s) and other factors in this electron transport system has been studied by other workers too (4, 7). This results were confirmed and extended by this study. The results described above may be summarized as in Fig. 4.

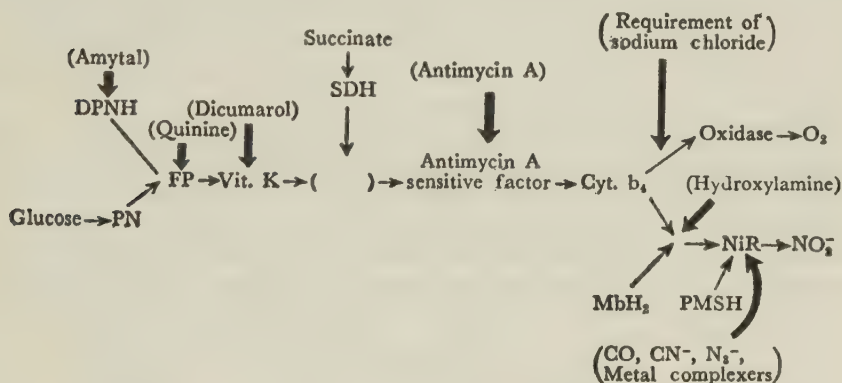


FIG. 4. Postulated scheme of electron transport system to nitrite*.

Lack of salt effect on $\text{MbH}_2\text{-NiR}$ system suggests that these dyes react more directly with NiR without participation of some intermediate electron carriers than with cytochrome b_4 . Requirement of high salt concentration of DPNH-NiR system may be attributed to the halophilic nature of the cyt. $b_4\text{-NiR}$ step.

The inhibition by amytal suggests the participation of pyridine nucleotide (18) in glucose-NiR system. Mode of action of quinine was not clarified sufficiently, but it has been known to inhibit the flavin enzyme system (19, 20). The inhibition by quinine of glucose-NiR system suggests the participation of flavin or some electron carrier presumably relating to flavin.

Lack of amytal, quinine and dicumarol inhibition in succinate-NiR system can be explained by Fig. 4. The heme of cytochrome b_4 is not a protoheme (21) but it is known that a cytochrome containing protoheme

* Arrows indicate the possible site of action of reagents. Abbreviations; PN, pyridine nucleotide; FP, flavo-protein; SDH, succinic dehydrogenase; vit., vitamin.

exists in the particulate fraction of the bacterium¹⁾. Close similarity between the electron transport system functioning in the reduction of nitrite and the mammalian terminal oxidase system suggests a possibility that a cytochrome containing protoheme participates at the place shown with the blank parentheses in Fig. 4. Inhibition of electron transport systems by antimycin A has not yet been reported in bacteria. But in this bacterium, electron transport system leading to nitrite was inhibited by relatively high concentrations of antimycin A (0.5–1.0 $\mu\text{g./mg.}$ dry weight).

Yamada and Virtanen (22) reported that the same bacterium produced nitrous oxide from nitrite. In this experience, over 95 per cent of the total volume of the gas produced from nitrite was nitrogen²⁾. Probably in Yamada's case the bacterium lacks some components or has some additional components of the denitrifying system.

Iwasaki and Mori (8) reported that the mechanism of denitrification in their denitrifying pseudomonad was an amine-nitrite reaction. Our results differ from their results in the existence of inhibition by hydroxylamine, carbon monoxide in the dark, and by nitric oxide, and the lack of inhibition by SH-reagent. It may be probable that different mechanisms are participating in denitrification in different organisms.

This bacterium contains a powerful hydroxylamine reductase activity (10), but nitrite is not reduced to hydroxylamine and ammonia. Hydroxylamine reductase was also found in other denitrifying bacteria by several workers (17)³⁾⁴⁾. The existence of some relation between HdR and denitrification process and the separation of the nitrite reductase system into soluble and particulate components will be reported in the next paper.

SUMMARY

1. Electron transport system functioning in the reduction of nitrite and the properties of NiR were studied using a halotrelant *Micrococcus* (strain 203) as the material.

2. Ferrocytochrome b_4 reduced nitrite in the presence of NiR. The reaction required high salt concentrations.

3. Electron transport from DPNH to nitrite, requiring high salt concentrations, was activated by FAD about two-fold, and inhibited by antimycin A.

4. Glucose-NiR system was inhibited by amytal, quinine, dicumarol and antimycin A, and activated by FAD and menadione.

1) Personal communication from Prof. T. Mori of the Department of Biology, Nagoya University.

2) Kindly analysed by Prof. T. Koyama of the Water Research Institute, Nagoya University, to whom author's thanks are due.

3) Personal communication from Dr. H. Iwasaki of the Department of Biology, Nagoya University.

4) Personal communication from Dr. M. Kamen of Brandeis University.

5. Succinate-NiR system was inhibited by antimycin A but not inhibited by amytal, quinine and dicumarol.

6. NiR was inhibited by cyanide, azide and metal chelators. The carbon monoxide inhibition in the dark was not reversed by light. Hydroxylamine inhibited the MbH₂-NiR system but did not inhibit the PMSH-NiR system. The nitric oxide inhibition at 0.1 atm. was stonger in MbH₂-NiR system than in PMSH-NiR system.

7. The similarity of the electron transport system investigated to the mammalian terminal oxidase system was discussed.

8. The electron transport system functioning in the reduction of nitrite may by summarized as shown in Fig. 4.

The author wishes to thank Prof. F Egami for his kind encouragement and guidance, and Dr. S. Taniguchi, Mr. K. Ohmachi and Mr. E. Itagaki for their helpful discussion. He also wishes to express his thanks to Prof. T. Mori and Dr. H. Iwasaki of the Department of Biology, Nagoya University for their helpful suggestion.

REFERENCES

- (1) Taniguchi, S., Sato, R., and Egami, F., A Symposium on Inorganic Nitrogen Metabolism, The Johns Hopkins Press, Baltimore, p. 87 (1956)
- (2) Nason, A., A Symposium on Inorganic Nitrogen Metabolism, The Johns Hopkins Press, Baltimore, p. 101 (1956)
- (3) Taniguchi, S., Asano, A., Iida, K., Kono, M., and Egami, F., Proceedings of International Symposium on Enzyme Chemistry, Maruzen, Tokyo, p. 238 (1958)
- (4) Allen, M. B., and van Niel, C. B., *J. Bacteriol.*, **61**, 397 (1952)
- (5) Sacks, L. E., and Barker, H. A., *J. Bacteriol.*, **64**, 247 (1952)
- (6) Nason, A., Abraham, R. G., and Averbach, B. C., *Biochim. et Biophys. Acta*, **15**, 159 (1954)
- (7) Kluyver, A. J., and Verhoeven, W., *Antonie van Leeuwenhoek J. Microbial. Serol.*, **20**, 241 (1954)
- (8) Iwasaki, H., and Mori, T., *J. Biochem.*, **42**, 375 (1955); Iwasaki, H., Matsubayashi, R., Mori, T., *J. Biochem.*, **43**, 295 (1956); Iwasaki, H., and Mori, T., *J. Biochem.*, **45**, 133 (1958)
- (9) Medina, A., and Nicholas, D. J. D., *Nature*, **171**, 533 (1957)
- (10) Kono, M., Taniguchi, S., and Egami, F., *J. Biochem.*, **44**, 615 (1957)
- (11) Novak, R., and Wilson, P. W., *J. Bacteriol.*, **55**, 517 (1948)
- (12) Singer, T. P., and Kearney, E. B., Methods of Biochemical Analysis, Interscience Publishers, Inc., New York., Vol. **4**, p. 312 (1957)
- (13) Yamada, T., Asano, A., *Ccmpt. rend. soc. biol.*, **143**, 1310 (1954)
- (14) Potter, V. R., and Reif, A. E., *J. Biol. Chem.*, **194**, 287 (1952)
- (15) Nason, A., and Lehman, I. R., *J. Biol. Chem.*, **222**, 511 (1956)
- (16) Martius, C., and Märki, F., *Biochem. Z.*, **329**, 449 (1956)
- (17) Verhoeven, W., and Takeda, Y., A Symposium on Inorganic Nitrogen Metabolism, The Johns Hopkins Press, Baltimore, p. 159 (1956)
- (18) Chance B., Enzymes; Units of Biological Structure and Function, Academic Press, New York, p. 447 (1956)
- (19) Burton, K., *Biochem. J.*, **48**, 458, (1956)

- (20) Eichel, H. J., *Biochim. et Biophys. Acta*, **22**, 571, (1956)
- ((21) Egami, F., Itahashi, M., Sato, R., and Mori, T., *J. Biochem.*, **40**, 527 (1953)
- (22) Yamada, T., and Virtanen, A. I., *Acta Chim. Scand.*, **10**, 20 (1956)

EFFECT OF 6-AZAURACIL ON CELLS AND SUBCELLULAR PREPARATIONS OF *ESCHERICHIA COLI**

By NOZOMU OTSUJI AND YASUYUKI TAKAGI

(From the Department of Biology, Faculty of Science and the Department of Biochemistry, Medical School, Osaka University, Osaka)

(Received for publication, October 30, 1958)

Previous studies with the uracil analog, 6-azauracil (*as*-triazine-3, 5-dione, AzU) have demonstrated its ability to inhibit growth of various microorganisms and of several experimental neoplasms in mice (2-5). Studies on nucleoside and nucleotide metabolites of the analog isolated from the culture medium or from the inhibited cells suggested that AzU might be first converted to phosphorylated derivatives of its riboside (6-azauridine) in order to become an effective inhibitor of growth (6-9). Recently it was found that orotic acid, orotidine 5'-phosphate and uridine 5'-phosphate accumulate in the culture medium of *Escherichia coli* B inhibited by AzU (10, 11), and further evidence was obtained that a site at which the analog acts following its conversion to nucleotide derivatives is orotidylic acid decarboxylase (12).

However it is likely that numerous other effects of AzU on bacteria may be produced as a consequence of the primary ribotidation. The work to be reported here concerns the antibacterial action of the analog on *Escherichia coli* K-12. It is suggested from experimental data that the inhibitory action of AzU on the growth of this organism is not necessarily a result of incorporation of the unnatural base into nucleic acids, but is more probably due to interference with utilization or function of uridine nucleotide compounds, especially in the synthesis of the cell wall.

MATERIALS AND METHODS

Materials—AzU was generously provided by Dr. Arnold D. Welch of Yale University, and *N*-acetylglucosamine used as standard in the assay was a gift of Dr. J. L. Strominger of Washington University.

Crystalline lysozyme was obtained from egg-white as described by Alderton and Fevold (13).

The bacteria were grown, with shaking, for 18 hours at 37° in a salt-glucose synthetic medium. At the end of this period, after diluting the entire culture in fresh medium, the cells were reincubated for an additional 2 hours with vigorous aeration. The protoplast or lysed protoplast preparations were prepared from the rejuvenated cells by a modification** of the procedure used by Repaske (14) and Spiegelman (15).

* A preliminary report of this work was given previously (1).

** Otsuji, N., and Takagi, Y., unpublished data.

Analytical Methods—The synthesis of tryptophanase was followed by the formation of indole, which was colorimetrically determined by Ehrlich's aldehyde reagent according to the procedure of Goto (16). Protein was measured, after trichloroacetic acid precipitation, by the Folin method (17). RNA and DNA were assayed by the orcinol, and diphenylamine methods respectively, after extraction from perchloric acid precipitates of bacteria. *N*-Acetylamino sugar esters were determined using the method described by Strominger (18).

RESULTS

Inhibitory Effect on Growth of E. coli—Exposure of *E. coli* in the salt-glucose medium to 1 μ g. per ml. of AzU caused complete inhibition of growth. Alterations in the bacteria with respect to nucleic acid and protein synthesis in the presence of AzU at a level of 10 μ g. per ml. are illustrated in Table I. As determined by measurements of turbidity, there was slight growth during the initial 30 minutes only. It was found that while the growth of the organisms was strongly inhibited by the presence of AzU, protein synthesis was unaffected or even slightly stimulated as compared with that of an uninhibited culture during the test period. A normal increases in RNA was also observed during the first 30 minutes, though later it tended to be slightly depressed. DNA formation did not differ much from that in

TABLE I

Short Term Effects of Azauracil on the Growth of E. coli

The rejuvenated bacteria as described in the text were resuspended in a similar fresh medium with or without azauracil, to give an optical density of 0.1 at 660 $m\mu$. After 60 minutes at 37°, aliquots of bacterial suspension were taken for turbidity, nucleic acid and protein assays. The results are expressed as changes in optical density at the respective wavelengths per 5 ml. of the culture for DNA, and per 1 ml. for protein and RNA.

Addition	Turbidity (O.D. 660 $m\mu$)	Diphenylamine reaction (O.D. 600 $m\mu$)	Orcinol reaction (O.D. 660 $m\mu$)	Folin method (O.D. 500 $m\mu$)
None	+0.081	+0.074	+0.730	+0.11
Azauracil (10 μ g./ml.)	+0.013	+0.054	+0.700	+0.124

the control for about 30 minutes and then ceased abruptly in contrast to a culture not exposed to the analog.

Loss of viability of the cells during exposure to AzU was measured by plating. However, values found for cultures at the start of the incubation and after treatment were the same.

Effect on the Enzyme Formation—The results described above are of interest, because nucleic acid and protein increased under conditions which inhibited growth of the cells. At present there is little doubt that RNA formation is

involved in some way in synthesis of new protein. To exclude the possibility, that in AzU-treated cells the abnormal protein is produced as a consequence of formation of abnormal nucleic acid containing the analog, some measure of the physiological activity was needed. A study of enzyme induction was therefore made.

When *E. coli* cells harvested from salt-glucose medium were incubated with tryptophan as inducer, synthesis of tryptophanase was induced after a lag phase of 30 to 60 minutes and increased rapidly with time. The effect of the presence of AzU, at various concentrations, on tryptophanase formation was studied. As shown in Table II, the analog, even at a level of 500 $\mu\text{g. per ml.}$, did not affect enzyme formation in contrast to its marked inhibitory action at low concentration on growth. It was suspected that a natural compound like tryptophan which may reach the locus of enzyme-forming system in the cell faster than AzU will prevent the inhibitory action. Therefore in the next experiments, the cells were exposed to AzU for

TABLE II

Effects of Azauracil on Tryptophanase Formation in various Preparations of E. coli

1.0 ml. of the cell suspension (optical density at 660 $m\mu$, 0.3) was incubated with 3.0 ml. of phosphate buffer (pH 7.7, 0.5 *M*) and 3.0 mg. of L-tryptophan in a total volume of 15 ml. The incubation mixture for the subcellular preparations contained additional supplements of 1.5 μM of ATP, 15 μM of hexose diphosphate, 25 mg. of the amino acid mixture and 1.5 gm. of sucrose. During incubation, aliquots of the mixture were analyzed at 30 minutes intervals for indole. The relative rate of tryptophanase formation at 2 hours is shown in the table, in which the amount of indole produced in the absence of the inhibitor is represented by 100.

Preparation	Addition	Concentration ($\mu\text{g./ml.}$)	Comparative rate of enzyme formation (per cent)
Cell suspension	None		100
	Azauracil	500	110
Protoplasts	None		100
	Azauracil	10	22
		100	0
Lysed-protoplasts	None		100
	Azauracil	1	42
		5	14
		10	6
		20	0

60 minutes in salt-glucose medium. After this period damage to the cells was established, and then tryptophan was added as inducer and the cells incubated for 3 hours. However, enzyme formation in AzU pretreated *E. coli* was again unaffected. These facts indicate that the synthesized RNA and protein of intact cells in the presence of AzU are normal and capable

of physiological functions. The data are in accord with the earlier results of Yates and Pardee, who observed that AzU inhibits β -galactosidase formation by *E. coli* B only slightly (19).

In the past few years many laboratories have reported the successful preparation of various subcellular fractions exhibiting protein-synthesizing capacity. When protoplast or lysed-protoplast preparations suspended in nutrient media containing ATP, hexose diphosphate and the amino acid

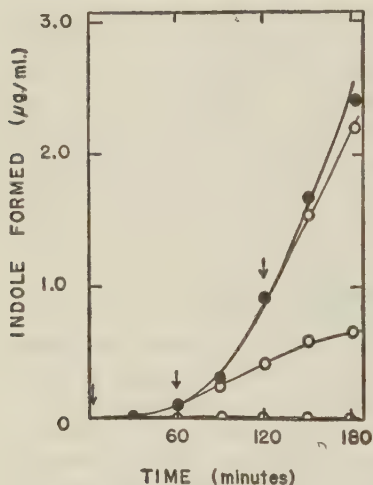


FIG. 1. Effects of azauracil on development of tryptophanase in lysed-protoplasts. Experimental procedure as described in Table II, except that azauracil was added (final concentration 20 μ g. per ml.) at the times indicated by arrows. The closed circles represent indole formation in the absence of inhibitor, and the open circles show its production after addition of the analog.

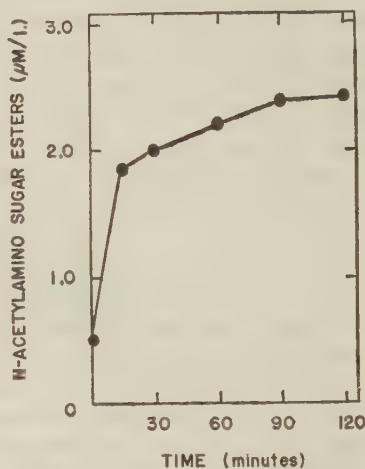


FIG. 2. Time-course of the azauracil-induced accumulation of *N*-acetyl amino sugar esters in *E. coli*. The rejuvenated organisms were suspended in 1.5 liter of salt-glucose medium containing 10 μ g. of azauracil per ml., to give an optical density of 0.16 at 660 $m\mu$, and incubated at 37° with aeration. Aliquots of 250 ml. were taken at intervals as shown and analyzed for *N*-acetyl amino sugar esters, which are expressed in the figure as μ M per liter of culture at an optical density of 0.5.

mixture plus stabilizing agent like sucrose were incubated in the presence of tryptophan as inducer, tryptophanase was induced after about 60 minutes lag phase. The effect of AzU on enzyme formation under these conditions was studied and found to be completely different from that observed in intact cells (Table II). Addition of even as little as 1 μ g. per ml. of the analog caused about 50 per cent inhibition of enzyme synthesis; with higher concentrations complete cessation was observed. If AzU was added after the inducer, at a time when enzyme formation had already occurred, an inhibition of further enzyme synthesis was achieved and consequently the

production of indole proceeded linearly after the addition of the analog (Fig. 1). While in subcellular preparations obtained from AzU-resistant *E. coli*, a block in enzyme formation was not observed on exposure to AzU. The results may be explained by the earlier observation that resistance to AzU is accompanied by a parallel loss of ability to convert AzU to the riboside and other metabolites (8).

Accumulation of N-Acetylmino Sugar Esters—The data so far mentioned suggest that the inhibitory action of AzU on intact cells is neither due to leakage of the cell contents brought about by damage of the cell membrane or wall, nor to abnormal nucleic acid and protein formation. Therefore the synthetic process necessary to support the cell wall was next studied. It has been demonstrated that there is accumulation of uridine diphosphate *N*-acetylmino sugar compounds in penicillin-inhibited *Staphylococcus aureus*, which Strominger recently presumed to be a precursor of the bacterial cell wall (20, 21). Thus experiments were designed to test the effect of AzU on the amount of *N*-acetylmino sugar esters. As indicated in Fig. 2, the level of these compounds in the acid-soluble fraction of cells incubated with AzU for 1.5 hours increased about 6-fold over that of a control uninhibited

TABLE III
*Influence of various Conditions on Accumulation of
N-Acetylmino Sugar Esters*

Washed organisms were resuspended in 250 ml. of a salt medium containing the supplements or analogs shown below at pH 7.0. All cultures were incubated at 37° with aeration for 2 hours, and analyzed for *N*-acetylmino sugar-containing compounds.

No. of experiment	Supplements, (per liter)	Analog, (per ml.)	μM per liter of culture at optical density 0.5 (660 $m\mu$)
1.	None	Azauracil 10 μg .	0.61
	Amino acid mixture 2 g.	Azauracil 10 μg .	1.26
	Glucose 3 g.	Azauracil 10 μg .	3.56
2.	Glucose 3 g.	None	0.42
	Glucose 3 g.	6-Mercaptopurine 10 g.	0.45
	Glucose 3 g.	Azauracil 10 μg .	2.48

culture. The low concentration of AzU which produced inhibition of growth induced an accumulation of nucleotides. The degree of the AzU-induced accumulation was dependent upon the experimental conditions (Table III); it was negligible with washed cells of *E. coli* suspended in a salt medium, and the addition of the amino acid mixture induced a small accumulation. Supplementation with glucose produced a marked effect. Actually, the ac-

cumulation of *N*-acetyl amino sugar compounds began promptly upon the addition of AzU, and the time for 80 per cent of maximal accumulation was of the order of 30 minutes after the addition (Fig. 2). At this time cessation of growth and synthesis of DNA by AzU treated cells showed the damage of all the cells. These findings indicate that exposure of the growing cells to AzU resulted in the accumulation of esterified *N*-acetyl amino sugars.

6-Mercaptopurine, which exhibited a similar inhibitory action on the growth of *E. coli* at a level of 1 μ g. per ml., was found not to cause accumulation of these compounds (Table III). Therefore it appears unlikely that these cell wall precursors accumulate whenever cell metabolism is disturbed by antimicrobial agents.

DISCUSSION

Several points regarding the precise mechanism of action of AzU are worthy of further discussion. It has been generally presumed that the base analog is incorporated into unnatural nucleic acid, in which the normal base is replaced by the analog, with a consequent loss of the function. The observation of the rapidity of the effect of AzU in subcellular systems, combined with the results obtained from the AzU-resistant cells, may suggest the same kind of action of AzU. However this appears not to be the main cause of the antibacterial action, since exposure of the cells to the analog does not affect RNA and protein synthesis in contrast to its marked inhibitory action on growth. Thus AzU has been shown to have an inhibitory action which differs from the general one found in the analog.

It has been well established that the nucleotides occurring in the acid-soluble fraction possess coenzyme functions that are superimposed upon their functions as precursors of nucleic acids. Therefore it is likely that analog-containing nucleotide derivatives competitively antagonize utilization or function of the corresponding native nucleotide compounds in coenzyme functions as well as in nucleic acid synthesis. Furthermore coenzyme activities may be more sensitive to the analog than the latter role as the precursor. Some evidence supporting the idea has been reported already for 8-azaguanine (22). Hence an attractive possibility for consideration as the mechanism of inhibitory action of AzU on intact cells, is that the uptake of the analog may be limited considerably and small amounts of AzU-containing nucleotide derivatives formed the invading analog will preferentially block some metabolic pathway, which is essential to cell multiplication. When the subcellular preparations are exposed to AzU, much analog will penetrate without limitation into the preparations and produce inhibition of both coenzyme functions and precursor role, resulting in complete cessation of enzyme formation. The demonstration of the accumulation of *N*-acetyl amino sugar esters favors this assumption.

The rapidity of the response to the analog and its relation to the threshold concentration for growth inhibition suggest that nucleotide ac-

cumulation is not a secondary effect of AzU. It needs caution to interpret this observation, since several laboratories have obtained the evidence of increased levels of amino sugar derivatives in the cultures inhibited with a wide variety of agents including aminopterin and chloramphenicol as well as penicillin. However it might be proposed that the accumulation by these agents is due to indirect effects and the results described above suggest the interference of AzU with cell wall synthesis. Nevertheless great difficulty has been experienced in fractionating the acid-soluble fraction to obtain the AzU-containing nucleotides, owing to small amount of these compounds. At present, it is questionable whether experiments can be carried much further.

E. coli treated with AzU were examined with a phase-contrast microscope, but it was not possible to see gross changes in shape or size. It is presumed that the rapidity of the inhibitory action on cell wall formation probably leads to subsequent depression of cell multiplication, without any great change in the appearance of the bacteria.

SUMMARY

Exposure of *Escherichia coli* K-12 in salt-glucose medium to 1 μ g. per ml. of AzU caused inhibition of growth. A study was made of alterations in the bacteria in the presence of AzU at a level of 10 μ g. per ml. It was found that 30 minutes after addition of AzU synthesis of DNA stopped while the RNA and protein content of the bacteria increased normally beyond this time even in the presence of a growth inhibitory concentration of the analog. Tryptophanase induction in intact cells was unaffected by the presence of a high concentration of AzU, but that in subcellular preparations was strongly inhibited by the analog. Treatment of the cells with AzU under conditions of growth results in accumulation of esterified *N*-acetyl amino sugars. The results are discussed in terms of the competitive antagonism of the AzU-containing nucleotide derivatives to uridine-nucleotide compounds in cell wall synthesis.

The authors wish to express their appreciation to Prof. K. Ichihara and Prof. H. Kikkawa for their interest and encouragement in this work. This investigation was aided by a grant from the Ministry of Education.

REFERENCES

- (1) Takagi, Y., and Otsuji, N., *Biochim. et Biophys. Acta*, **29**, 227 (1958)
- (2) Handschumacher, R. E., and Welch, A. D., *Cancer Research*, **16**, 965 (1956)
- (3) Hakala, M. T., Law, L. W., and Welch, A. D., *Proc. Am. Assoc. Cancer Research*, **2**, 113 (1956)
- (4) Šorm, F., and Škoda, J., *Collection, Czechoslov. Chem. Commun.*, **21**, 487 (1956)
- (5) Šorm, F., Jakubovič, A., and Slechta, L., *Experientia*, **12**, 271 (1956)
- (6) Škoda, J., Hess, V. F., and Šorm, F., *Experientia*, **13**, 150 (1957)
- (7) Handschumacher, R. E., *Fed. Proc.*, **16**, 191 (1957)
- (8) Handschumacher, R. E., *Biochim. et Biophys. Acta*, **23**, 428 (1957)

- (9) Schindler, R., and Welch, A. D., *Science*, **125**, 548 (1957)
- (10) Šokoda, J., and Šorm, F., *Biochim. et Biophys. Acta*, **28**, 659 (1958)
- (11) Handschumacher, R. E., *Nature*, in press
- (12) Handschumacher, R. E., and Pasternak, C. A., *Biochim. et Biophys. Acta*, in press
- (13) Alderton, G., Fevold, H. L., *J. Biol. Chem.*, **164**, 1 (1946)
- (14) Repaske, R., *Biochim. et Biophys. Acta*, **22**, 189 (1956)
- (15) Spiegelman, S., *The Chemical Basis of Heredity* (W. D. McElroy and B. Glass, eds.), The Johns Hopkins Press, Baltimore, p. 232 (1957)
- (16) Goto, S., *J. Osaka Med. Assoc. (Japanese)*, **37**, 2413 (1938)
- (17) Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., *J. Biol. Chem.*, **193**, 265 (1951)
- (18) Strominger, J. L., *J. Biol. Chem.*, **224**, 509 (1957)
- (19) Yates, R. A., and Pardee, A. B., *J. Biol. Chem.*, **227**, 677 (1957)
- (20) Strominger, J. L., *J. Biol. Chem.*, **224**, 525 (1957)
- (21) Park, J. T., and Strominger, J. L., *Science*, **125**, 99 (1957)
- (22) Mandel, H. G., *Arch. Biophys.*, **76**, 230 (1958)

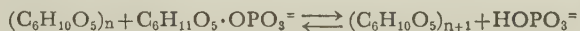
DETERMINATION OF PHOSPHORYLASE ACTIVITY IN THE PRESENCE OF β -AMYLASE

BY MICHINORI NAKAMURA

(From the Department of Agricultural Chemistry, the University of
Tokyo, Bunkyo-ku, Tokyo)

(Received for publication, November 4, 1958)

Phosphorylase adds reversibly glucosyl groups from α -D-glucose-1-phosphate (G-1-P) to the non-reducing ends of the primer oligo- and polysaccharide, forming α -1,4-glucosidic bonds and thus lengthening the amylose chain of the primer in such a way as can be formulated in the following equation (1):



For the determination of phosphorylase activity, inorganic phosphate, liberated from G-1-P in the catalyzed reaction, is usually taken as a measure of its activity (2). However, little or almost no liberation of inorganic phosphate is observed with extracts from some plant materials such as sweet potato and barley in spite of the presence in the extract of phosphorylase. This is because these extracts contain large amounts of β -amylase (3, 4) which degrades the primer polysaccharide added to the reaction digest ultimately to β -amylase-limit dextrin which has very weak or no priming activity (4, 5). When β -amylase activities are not so potent, phosphorylase activity can be determined by estimating inorganic phosphate liberated in the reaction, in which a large amount of primer polysaccharide is used and the order of the addition of the components to the reaction mixture is so changed as the enzyme solution is added at the final step to the preincubated mixture containing all the components except the enzyme (6).

β -Amylase activities in crude extracts from sweet potato and barley are, however, so potent that the liberation of inorganic phosphate in the reaction digest rapidly slows down and only a slight increase in inorganic phosphate with time is observed. That phosphorylase is actually present in these extracts was shown (i) either by partial purification of these extracts to remove a larger portion of β -amylase and give a preparation with a low activity ratio of β -amylase to phosphorylase (3, 4, 7) or (ii) by selective inhibition of β -amylase, thus enabling phosphorylase activity to be detected (8). The former method is unequivocal, but it has its disadvantage in that any purification procedure is always accompanied by some loss in enzyme activity, thus making quantitative determination of the enzyme activity in crude extracts impossible. In the latter method, phosphorylase activity can be detected in these extracts if a proper amount of mercuric chloride or

phenyl mercuric acetate is added to the enzymic digest, because β -amylase is more sensitive to mercury than is phosphorylase (8). By using a proper concentration of mercury, phosphorylase activities in these extracts can be determined semi-quantitatively. The highest enzymic activity obtained in a series of experiments with various concentrations of mercury can be regarded as a measure that at least the amount of phosphorylase corresponding to that activity is present in the enzyme solution used. The actual enzymic activity is, however, not known by this procedure, because phosphorylase is also sensitive, although less than β -amylase, to mercury and it is practically impossible to inhibit β -amylase completely without any concomitant inhibition of phosphorylase. It could be shown, however, by this procedure that phosphorylase activities were very potent in these extracts (9).

It would be possible, then, to determine phosphorylase activity quantitatively in the presence of β -amylase, were it possible to know how much phosphorylase is inhibited by mercury under the conditions used. The phosphorylase activity determined in the presence of a specified concentration of mercury and corrected for the inhibited activity gives a true value in these extracts. For this purpose, a known amount of partially purified potato phosphorylase was added to these extracts. The increase in phosphorylase activity of such extracts due to the addition of a known amount of phosphorylase in the presence of the same concentration of mercury can be measured. Thus, it is possible to show how much phosphorylase is inhibited by mercury under the specified conditions.

EXPERIMENTAL

Materials

α -D-Glucose-1-phosphate (G-1-P)—Crystalline potassium salt of G-1-P was prepared and purified essentially by the method of Hassid and McCready (10) with some modifications (11).

Partially Purified Potato Phosphorylase—Heat-treated potato-cyanide extracts, prepared essentially by the method of Green and Stumpf (12), were fractionated with ammonium sulfate according to Hidy and Day (13). Throughout the whole procedure, the pH of the enzyme solution was kept at 6.6–6.8 by the addition of dilute ammonia. The final product was stored in the ice-box as a suspension in a solution of ammonium sulfate of specific gravity 1.135 or in a deep freezer at -20° without loss in enzymic activity for several months.

Partially Purified Sweet Potato β -Amylase—The purification procedures by the method of Balls, *et al.* (14) were followed up to the stage of the “purified paste”.

Soluble Starch—This was prepared from commercial potato starch by the method of Lintner (15).

Sweet Potatoes—Various varieties of sweet potatoes were obtained through the courtesy of Mr. Masatoshi Onoda of the Chiba Branch Station, Kantô-Tôzan National Agricultural Experiment Station. In the preliminary tests, sweet potatoes bought from the market were used. Sweet potatoes were peeled and ground with a porcelain grinder. The brei was pressed through linen cloth. The pressed juice, after centrifuging to remove starch and cell debris, was stored in an ice-box without loss in enzymic activities for

several days.

Other reagents are commercial products.

Procedures

Determination of Phosphorylase Activity—The method reported previously (16) was used with some modifications. One unit is defined as the amount of enzyme which liberates 0.1 mg. of inorganic phosphorus in three minutes at 37° in a reaction mixture containing 1.0 ml. of 0.5 *M* acetate or maleate buffer at pH 6.0, 0.5 ml. of 5 per cent soluble starch, 0.5 ml. of 0.2 *M* G-1-P, and an appropriate amount of enzyme in a total volume of 3.5 ml.

Determination of β -Amylase Activity—The method used was reported previously (16). The reaction mixture contained, in a total volume of 20 ml., 1.0 ml. of 0.5 *M* acetate buffer at pH 5.0, 10 ml. of 2 per cent soluble starch and an appropriate amount of enzyme. One unit was defined as the amount of enzyme which liberates 1 mg. of maltose in 1 minute at 37° under the conditions given above.

RESULTS

Determination of Phosphorylase Activity in a Mixture of Partially Purified Preparations of Phosphorylase and β -Amylase—A representative result is given in Table I. In these experiments, solutions of phosphorylase (1.19 units/ml.) and β -amylase (16.0 units/ml.) were prepared by diluting stock solutions of the respective enzymes. One half ml. of the mixed enzyme solution contained 0.3 ml. of phosphorylase and 0.2 ml. of β -amylase.

To each of five small test tubes are pipetted buffer, water, enzyme solutions, and mercuric chloride in the amounts and in the order given in Table I, A. The total volume of each tube will be 2.5 ml. The tubes are incubated for 20 minutes at room temperature and then for further 20 minutes in a well-regulated water bath at $37 \pm 0.02^\circ$. The reaction is started by adding to the preincubated mixtures 0.5 ml. each of 0.2 *M* G-1-P and 5 per cent soluble starch solutions, both of which are maintained at the same temperature. Immediately after the start of the reaction and exactly after 10 and 20 minutes, 0.5 ml. aliquots are withdrawn from each tube and treated with 2.0 ml. of 6 per cent perchloric acid to stop the reaction. Aliquots of the deproteinized filtrates are used for the determination of inorganic phosphorus. Increases in inorganic phosphorus in each tube during 10 and 20 minutes incubations are obtained from these determinations. Average values for 10 minutes are used for the calculation. From the values for the tubes 2 and 3, and those for the tubes 4 and 5, increases in inorganic phosphorus due to added phosphorylase will be known. These values, when compared with the value for tube 1, will give the recovery of added phosphorylase under the specified concentrations of mercury. When it is assumed that phosphorylase in the mixed enzyme solution is inhibited to the same degree as the added phosphorylase, the corrected values for tubes 2 and 4 for the recovery of added phosphorylase under the specified conditions will give the actual phosphorylase activities in the mixed enzyme solution. Results are shown in Table I, B.

TABLE I

Determination of Phosphorylase Activity in Mixtures of Partially Purified Preparations of Phosphorylase and β -Amylase

A. Preincubation mixtures					
Tube no.	0.5 M Acetate buffer at pH 6.0 (ml.)	Distilled water (ml.)	Purified potato phosphorylase (ml.)	Mixed enzyme solution ¹⁾ (ml.)	2.5×10^{-5} M HgCl_2 (ml.)
1.	1.0	1.20	0.3	—	—
2.	1.0	0.75	—	0.5	0.25
3.	1.0	0.45	0.3	0.5	0.25
4.	1.0	0.67	—	0.5	0.33
5.	1.0	0.37	0.3	0.5	0.33

B. Results							
Tube no.	Δ_{10} Pi (mg.)	Δ_{20} Pi (mg.)	Av. Δ_{10} Pi (mg.)	Increase in Pi due to added phosphorylase (mg.)	Recovery of added phosphorylase (%)	Corrected for inhibition (mg.)	Phosphorylase activity (units/ml.)
1.	0.118	0.238	0.119				1.19
2.	0.064	0.127	0.064			0.119	1.19 ²⁾
3.	0.127	0.258	0.128	0.064	54.0		
4.	0.053	0.099	0.052			0.113	1.13 ²⁾
5.	0.109	0.210	0.107	0.055	46.2		

1) The mixed enzyme solution (0.5 ml.) contains 0.3 ml. of phosphorylase and 0.2 ml. of β -amylase.

2) Expressed in units per ml. of the phosphorylase solution used to make the mixed enzyme solution.

In ten cases of such experiments, with various combinations of phosphorylase and β -amylase, the recoveries of phosphorylase from the mixed enzyme solutions were: 110, 107, 97, 95, 98, 93, 108, 111, 107, and 99 per cent. Thus, it may be said that this procedure gives the value of phosphorylase in the presence of a large amount of β -amylase with errors seldom exceeding 10 per cent. With a very proper concentration of mercury, recovery of added phosphorylase as high as 96 per cent was obtained. In such a case, phosphorylase activity is determined with an error of less than 3 per cent. When the recoveries of added phosphorylase are low, the errors inherent in the determination of inorganic phosphorus will influence greatly the subsequent calculations and large errors will result. In such cases, it is recommended that determinations may be repeated again with different concentrations of mercury.

Determination of Phosphorylase Activities in Crude Extracts from Sweet Potatoes—The determination of phosphorylase activities in crude extracts

from sweet potatoes was carried out in the same way as has already been given in the preceding section, except that the extracts were used as the source of enzyme in place of the "mixed enzyme solution". For the purpose of quantitative determination, purified phosphorylase from sweet potatoes, instead of purified potato enzyme, must be used in recovery experiments, because it has not been established whether phosphorylases from both sources have the same sensitivity to mercury. In these experiments, however, partially purified potato phosphorylase was used, because it is intended in this paper to give procedures and examples and not quantitative data, and, moreover, it is not an easy matter to prepare highly purified phosphorylase from sweet potatoes totally devoid of β -amylase activity. The concentration of mercury in the preincubation mixtures was, instead of $(2\sim3)\times 10^{-6} M$ in most experiments with the mixed enzyme solutions, increased to $(7\sim 10)\times 10^{-8} M$ in most experiments with 0.5 ml. of five times diluted sweet potato extracts as the enzyme solution. Greater protein content of the sweet potato extract than that of the mixed enzyme solution necessitated the use of higher concentration of mercury for the proper inhibition of β -amylase in the extract.

TABLE II

Phosphorylase and β -Amylase Activities of Crude Extracts from Some Varieties of Sweet Potatoes

Sample	Phosphorylase activity (units/ml.)	β -Amylase activity (units/ml.)
Nôrin, No. 1.	16.9	137
Nôrin, No. 2.	17.6	722
Nôrin, No. 5.	23.3	336
Kantô, No. 35.	19.2	203
Kantô, No. 45.	14.8	128
Kantô, No. 46.	20.1	194
Kantô, No. 47.	16.4	167
Okinawa, No. 100.	17.8	529
Shirosengan	10.6	184
Okimasari	19.7	793

In Table II are given results of some determinations of phosphorylase activities in crude extracts from some varieties of sweet potatoes. These experiments were intended only to give some examples and each figure has no statistical significance. Nevertheless, it will be seen in the table that phosphorylase as well as β -amylase is very potent in sweet potatoes. The comparable values for potatoes are 9~10 units/ml. of pressed juice.

Inhibitors Other Than Mercury—Phenyl mercuric acetate was as effective as mercuric chloride in the procedures given above. In a search for a more

specific inhibitor for β -amylase, some reagents were tested but without success. A brief account of some of them is given here.

Ascorbic acid is a fairly potent inhibitor of β -amylase (17), but in concentrations which give a nearly complete inhibition of β -amylase it exhibits also a serious suppression of phosphorylase activity. The concentrations of ascorbic acid which give 50 per cent inhibition are approximately $1.4 \times 10^{-4} M$ for β -amylase and $2.5 \times 10^{-4} M$ for phosphorylase. It is, therefore, very difficult to find out a proper concentration of ascorbic acid which gives a nearly complete inhibition of β -amylase with as little inhibition as possible of phosphorylase. In most trials, the recoveries of added phosphorylase seldom exceeded 20 per cent.

Monoiodoacetate, up to 0.01 *M*, had only a slight inhibitory effect on both phosphorylase and β -amylase.

Sodium cyanide, in 0.1 per cent solution, was reported by Roy and Underkofler (18) to inhibit β -amylase completely in 1 hour at 30°. However, it was not attempted here to use sodium cyanide as a selective inhibitor of β -amylase, because it was shown previously (19) that cyanide ion had no inhibitory effect on β -amylase and the pH of 0.1 per cent aqueous solution of sodium cyanide (above 10) was deleterious not only to β -amylase but also to phosphorylase.

DISCUSSION

For the determination of phosphorylase activity, two assumptions have been made in the proposed procedure. First, the amount of protein in the phosphorylase solution to be added in the recovery experiments is very small and does not affect significantly the protein contents in the enzymic digests. As the inhibition of phosphorylase as well as of β -amylase by mercury is much affected by the amount of contaminating proteins (8), the protein content in the added phosphorylase solution must be as small as possible. Otherwise, the degree of inhibition of phosphorylase in the presence or in the absence of added phosphorylase is not the same, thus making corrections of inhibited activity by way of the recovery of added phosphorylase invalid.

Second, in the determination of phosphorylase activities in crude extracts from sweet potatoes, it was assumed that the sensitivity to mercury of phosphorylases from potatoes and sweet potatoes was the same. This assumption is not valid when there is no direct supporting evidence for this assumption, although the properties of phosphorylases from both sources are reportedly very similar (7, 20). This assumption may be avoided by using a purified preparation of phosphorylase from the same plant source, the phosphorylase activity of which is to be determined by this procedure, in the recovery experiments. For reasons given elsewhere, partially purified potato phosphorylase was used in these experiments. Therefore, the results obtained in Table II are, strictly speaking, not quantitative. Nevertheless, these values would be useful for comparative purposes.

Although the procedures described in this paper, when carried out with due precautions, permit quantitative determination of phosphorylase activity in the presence of potent β -amylase, this method has also its disadvantages, which, to mention a few, are as follows: (i) The most suitable concentration of mercury, which inhibits β -amylase as much as possible and, at the same time, inhibits phosphorylase as little as possible, must be searched for in preliminary experiments, because the concentration of mercury just sufficient to inhibit all the β -amylase present in the phosphorylase digest varies greatly from sample to sample by its protein content. This method is, therefore, somewhat time-consuming. (ii) Since phosphorylase activity is calculated by correcting the observed activity in the presence of mercury for the inhibited activity of added phosphorylase in the presence of the same concentration of mercury, small errors in the determination would give a large influence on the results obtained. (iii) It is, therefore, recommended to carry out determinations in duplicate, using five tubes simultaneously, to obtain a more reliable result.

Nevertheless, the method described in this paper is, as far as the author is aware, the only one which permits quantitative determination of phosphorylase activity in the presence of potent β -amylase and will be useful for that purpose.

SUMMARY

Detailed procedures are given for the quantitative determination of phosphorylase activity in the presence of potent β -amylase. This method is based on the finding that β -amylase is more sensitive to mercuric chloride than is phosphorylase. By correcting the observed phosphorylase activity in the presence of a suitable concentration of mercury for the recovery of added phosphorylase in the same digest, true phosphorylase activity is obtained. It was found by this method that crude extracts from sweet potatoes are very potent in phosphorylase activities.

The author wishes to express his sincere thanks to Prof. S. Funahashi for his interest in this work and to Mr. M. Onoda for the generous gift of various varieties of sweet potatoes.

REFERENCES

- (1) Hassid, W. Z., Doudoroff, M., and Barker, H. A., in *The enzymes*, ed. by Sumner, J. B., and Myrbäck, K., Academic Press, Inc., New York, Vol. I, part 2, p. 1014 (1951)
- (2) Whelan, W. J., in *Methods in enzymology*, ed. by Colowick, S. P., and Kaplan, N. O., Academic Press, Inc., New York, Vol. I, p. 192 (1955)
- (3) Porter, H. K., *Biochem. J.*, **47**, 476 (1950)
- (4) Nakamura, M., *Nippon Nôgei-kagaku Kaishi*, **24**, 302 (1951)
- (5) Proehl, E. C., and Day, H. G., *J. Biol. Chem.*, **163**, 667 (1946)
- (6) Nakamura, M., in *Kôso Kenkyu-hô*, ed. by Akabori, S., Asakura Shoten, Tokyo, Vol. II, p. 569 (1956)

- (7) Nakamura, M., *Nippon Nôgei-kagaku Kaishi*, **25**, 413 (1952)
- (8) Nakamura, M., and Maruo, B., *Nippon Nôgei-kagaku Kaishi*, **24**, 299 (1951)
- (9) Nakamura, M., and Toda, H., *Nippon Nôgei-kagaku Kaishi*, **25**, 248 (1952)
- (10) Hassid, W. Z., and McCready, R. M., *Biochemical preparations*, John Wiley & Sons, New York, Vol. **4**, p. 63 (1955)
- (11) Mori, K., and Nakamura, M., *Bull. Agr. Chem. Soc. Japan*, in press
- (12) Green, D. E., and Stumpf, P. K., *J. Biol. Chem.*, **142**, 355 (1942)
- (13) Hidy, P. H., and Day, H. G., *J. Biol. Chem.*, **160**, 273 (1945)
- (14) Balls, A. K., Walden, M. K., and Thompson, R. R., *J. Biol. Chem.*, **173**, 9 (1948)
- (15) Lintner, C. J., *J. prakt. Chem.*, **34**, 378 (1886)
- (16) Nakamura, M., Yamazaki, K., and Maruo, B., *Nippon Nôgei-kagaku Kaishi*, **24**, 197 (1951)
- (17) Purr, A., *Biochem. J.*, **28**, 1411 (1934)
- (18) Roy, D. K., and Underkofler, L. A., *Cereal Chem.*, **27**, 404 (1950)
- (19) Nakamura, M., *Science*, **117**, 629 (1953)
- (20) Nakamura, M., *Nippon Nôgei-kagaku Kaishi*, **26**, 260, 267 (1952)

LETTERS TO THE EDITORS

he Journal of Biochemistry, Vol. 46, No. 6, 1959

DECREASING EFFECT OF DEOXYCHOLIC ACID ON BLOOD CHOLESTEROL LEVEL OF RATS AND HUMANS

Since Schönheimer (1) first demonstrated, it has been generally known that the simultaneous ingestion of cholesterol and the bile acid increases the cholesterol level of the blood as well as of the liver of rat.

In such feeding experiments, however, the concentration of cholesterol in the diet was unphysiologically high (1 per cent) (2).

In order to get a further information about the above-mentioned effect of bile acid, several kinds of bile acid have been given to rats fed a cholesterol-free diet, and the following interesting results were observed.

Six groups of young male rats (body weight of about 115 g.) of the Wistar strain were used, each group consisting of 10 rats, and were fed a synthetic diet (casein 20 per cent; cellulose-agar powder 10 per cent; salt mixture (Mc Collum and Simmonds) 3 per cent; dry yeast 3 per cent; cane sugar 58 per cent; pea-nut oil 5 per cent) with or without bile acid.

When 80 mg. of each bile acid was ingested per rat daily, plasma cholesterol level of hyodeoxycholic acid group was increased in 5 days by 26 per cent, that of cholic acid group by 103 per cent, and that of chenodeoxycholic acid group by 123 per cent of the initial level, while that of the control animals remained unchanged. It was a striking fact that ingestion of deoxycholic acid, on the contrary, markedly reduced plasma cholesterol level to 65 per cent of the initial level in 5 days, and to 33 per cent in 10 days.

This remarkable effect of deoxycholic acid on plasma cholesterol was observed, even when the bile acid was ingested in an amount of as low as 10 mg. per rat daily.

The cholesterol concentration of the liver of these rats was found likewise to be reduced remarkably. By the courtesy of Dr. T a n a k a, Prof. of Pathology of our School, the histopathological investigation of the rats given 10 mg. of deoxycholic acid was performed, and all of their organs tested showed no pathological change.

Apocholeic acid, a compound closely related in structure to deoxycholic acid, was found likewise to have a decreasing effect on the cholesterol level of both the plasma and the liver of rats.

Some kinds of bile acid were given in dosis of 10 mg. per rat daily, and the results are presented in Table I.

Such an effect of deoxycholic acid as mentioned above was quite reproducible in humans. Six healthy men ingested 0.6 g. of deoxycholic acid daily for 8 days, and the initial cholesterol level of 160 mg./100 ml. (in average) was decreased in 5 days down to 110 mg./100 ml. (in average), to regain the normal level when the feeding of the bile acid was stopped.

TABLE I
Cholesterol Level of the Blood and the Liver of Rats Fed Some Bile Acids

Day Bile acid ingested	Before experiments Plasma cholesterol (mg./dl.)	Feeding of bile acid		
		5 days Plasma cholesterol (mg./dl.)	10 days Plasma cholesterol (mg./dl.) Liver cholesterol (mg. %)	
None	53±6.5	53±7.2	54±2.5	483±39.1
Cholic	60±6.8	105±7.3	101±6.3	651±25.4
Deoxycholic	55±3.4	40±2.9	38±1.4	395±22.9
Apocholic	53±5.8	46±2.5	44±2.7	412±18.1
Dehydrocholic	52±4.2	52±6.9	54±3.9	564±29.1

During the experiment, abnormalities were scarcely encountered in the blood and the urine samples of these volunteers, except that a very light leucopenia was observed in every case.

The above experiments could demonstrate clearly that deoxycholic acid (and apocholic acid) is a specific reagent which decreases the cholesterol level of the blood as well as of the liver. Such effect of deoxycholic acid could be interpreted in the following way: Deoxycholic acid seems to interfere with the biosynthesis of cholesterol in the liver far more strongly than cholic acid (2), since the former has been proved not to increase, but to depress the secretion of the total bile acid into bile more markedly than cholic acid (3).

REFERENCES

- (1) Schöheimer, R., *Biochem. Z.*, **258**, 147 (1924)
- (2) Pihl, A., *Acta Physiol. Scand.*, **34**, 206 (1955)
- (3) Ogura, M., Otagaki, H., Fujioka, T., and Kono, Y., to be published

Department of Biochemistry,
 Tottori University
 School of Medicine, Yonago

KAZUMI YAMASAKI
 KYUTARO SHIMIZU
 MAKOTO YASUMIZU
 TETSUHIKO SUGIHARA

(Received for publication, February 13, 1959)

LETTERS TO THE EDITORS

The Journal of Biochemistry, Vol. 46, No. 6, 1959

ON THE TRANSFORMATION OF DEHYDROCHOLIC ACID INTO DEOXYCHOLIC ACID IN THE RABBIT

An assumption has been advanced by the late Prof. T. Shimizu and his collaborators (1), that cholic acid is transformed through the liver into other bile acids, especially into deoxycholic acid, dehydrocholic acid being here postulated as an obligatory intermediate. Kim (2) administered cholic acid to guinea pig, whose bile contains no deoxycholic acid, and isolated the latter not only from the urine, but also from the bile. Lately Miyashita (3) in our laboratory has isolated deoxycholic acid from feces of rabbits injected intravenously with a large amount of dehydrocholic acid, and assumed that the former was derived from the latter. These findings were proposed to give additional evidences for Shimizu's assumption.

Recently Lindstedt and Sjövall (4), however, reported that C^{14} -cholic acid was transformed into C^{14} -deoxycholic acid during its enterohepatic circulation, and that the intestinal microorganisms, but not the liver, were responsible for this transformation.

Since C^{14} -dehydrocholic acid was now available (5), we attempted to re-examine Miyashita's experiment, using the labelled bile acid.

Administration of C^{14} -Dehydrocholate to the Rabbit with a Bile Fistula—Each of 3 rabbits was furnished with a bile fistula and then injected with C^{14} -dehydrocholate (100 mg./kg.; total radioactivity 419,862 c.p.m) intravenously. About 1 per cent of the radioactivity was recovered in the urine of 3 hours' urination after the injection, whereas none was present in the urine excreted thereafter. On the other hand, a large portion of the radioactivity appeared in the bile, and indeed, 50 per cent of the radioactivity injected was recovered within the first 30-minutes' period, and 10 per cent during the next 30-minutes'.

The bile was collected in a flask containing ethanol during the first 60-minutes' period after the injection of C^{14} -dehydrocholate and filtered. The ether extract of the filtrate (acidified) contained 24.5 per cent (in average) of all the radioactivity present in the bile.

The aqueous layer (pH 1) of the above extraction was extracted again with butanol (Norman (6)), and the extract contained practically all of the residual radioactivity of the bile.

The analysis of the C^{14} -bile acid contained in the ether and the butanol extracts was carried out separately, using the reversed-phase chromatography (7). Two peaks of radioactivity were observed on the chromatogram of each extract (peaks 1, 2, 3 and 4). Among them, reductodehydrocholic

(peak 2), glycoreductodehydrocholic (peak 3) and glycodehydrocholic (peak 4) acids were identified.

From its position on the chromatogram, the minor peak (peak 1) of the ether extract may neither be of deoxycholic nor of dehydrocholic acid.

These results clearly show that dehydrocholic acid passes through liver into bile, either being conjugated with glycine or partially reduced to reductodehydrocholic acid (conjugated or unconjugated), whereas its conversion into deoxycholic acid could not be confirmed.

Administration of C¹⁴-Dehydrocholate to the Intact Rabbits—Three intact rabbits were injected intravenously with C¹⁴-dehydrocholate (100 mg./kg.; total radioactivity 253,734 c.p.m.) and 4.5 per cent of the radioactivity was recovered in the urine of 3 hours' urination. This finding is consistent with the result of the dehydrocholate loading test (8).

Feces, collected for 3 days after the administration of labelled dehydrocholic acid and intestinal contents of the rabbits were combined, dried and extracted with ethanol.

The extract containing 15.1 per cent of the administered isotope was subjected to the chromatography (7) and revealed two bands of radioactivity. The major band was proved to be of the labelled deoxycholic acid. The identification was conducted as follows: 1. From the eluates corresponding to this band, crystals were obtained, which, on recrystallization from acetic acid, melted at 141° and showed no depression of the melting point on admixture with authentic acetic choleic acid (m.p., 145°); 2. one part of the above crystals was recrystallized with 120 mg. of unlabelled deoxycholic acid, and the recrystallized sample showed a specific activity of 24 c.m.p. which was found to remain unchanged on 4 further recrystallizations; 3. another part of the above crystals was rechromatographed with unlabelled deoxycholic acid and the peak of radioactivity coincided with the titration curve of deoxycholic acid.

Three days after the injection of labelled dehydrocholate, each animal was furnished with a bile fistula. The bile collected contained 27.1 per cent (in average) of the administered isotope. The butanol extract (6) of the bile was subjected to chromatography (7), and two bands of radioactivity were obtained. The major band was proved to be of glycodehydrocholic acid, and the minor one in a less polar region has not yet been identified.

The present experiments show that only the glycine-conjugation as well as the partial reduction of dehydrocholic acid takes place in the rabbit liver, and that the administered dehydrocholic acid was recovered from the feces as deoxycholic acid as previously assumed by Miyashita (3), and that contrary to Shimizu's assumption, the liver might have nothing to do with this transformation, instead, the intestinal microorganisms were probably responsible for this transformation as indicated by Lindstedt *et al.* (4).

REFERENCES

- (1) Shimizu, T., *J. Japan. Biochem. Soc.*, **20**, 118, 1948
- (2) Kim, C. H., *Z. physiol. Chem.*, **261**, 97 (1939)
- (3) Miyashita, N., *J. Yonago Med. Assoc.*, **4**, 160 (1953)
- (4) Lindstedt, S., and Sjövall, J., *Acta Chem. Scand.*, **11**, 421 (1957)
- (5) Bergström, S., Rottenberg, M., and Volts, J., *Acta Chem. Scand.*, **7**, 481 (1953)
- (6) Norman, A., *Acta Physiol. Scand.*, **32**, 1 (1954)
- (7) Bergström, S., and Sjövall, J., *Acta Chem. Scand.*, **5**, 1267 (1951); Sjövall, J., *Acta Physiol. Scand.*, **29**, 232 (1953)
- (8) Nakamura, T., *J. Japan. Biochem. Soc.*, **27**, 284 (1955)

Department of Biochemistry
Tottori University
School of Medicine, Yonago

KAZUMI YAMASAKI
TOHAKU WAKUTANI
HIROO TAKIMOTO
KYUTARO SHIMIZU

(Received for publication, March 26, 1959)

